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***Drosophila Melanogaster* as a Model for
Molybdo-Flavoenzyme Mediated Protection
against Chemical and Physical Stress**

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**Thesis submitted in partial fulfilment of the requirements for the
Degree of Doctor of Philosophy**

June 2012

Abstract

Aldehyde oxidase (AO) and xanthine oxidoreductase (XOR) are molybdo-flavoenzymes (MFEs) involved in the oxidation of hundreds of endogenous and exogenous aldehydes and N-heterocyclic compounds many of which are drugs, vitamins and environmental pollutants. Mutations in the XOR and molybdenum cofactor sulfurase (MCS) genes result in a deficiency of XOR or dual AO/XOR deficiency respectively. At present despite AO and XOR being classed as detoxification enzymes the definitive experimental proof of this has not been assessed in any animals thus far.

The aim of this project was to evaluate *ry* and *ma-l* strains of *Drosophila melanogaster* as experimental models for XOR and dual AO/XOR deficiencies respectively and to determine if MFEs have a role in the protection against chemical and physical stress. An additional aim was to determine the molecular basis for deficiency in *ma-l* strains by cloning and sequencing the MCS gene.

Spectrophotometric and HPLC assays demonstrated that *Drosophila* AO and XOR were able to catalyse the biotransformation of numerous substrates of the well-scrutinised mammalian orthologs. These included several aromatic aldehydes and N-heterocyclic pollutants, drugs and endogenous vitamins. Investigation of the enzyme activity in *ry* strain revealed a compromised ability to biotransform several XOR substrates that reflected the situation in human hereditary xanthinuria type I. Both *ma-l* strains were found to be unable to biotransform all AO substrates tested. These results confirmed that *Drosophila* were good experimental models for testing the role of MFEs as detoxification enzymes.

In order to test the role of the enzymes in chemoprotection, MFE substrates were administered to *Drosophila* in media and survivorship monitored. It was demonstrated that several methylated xanthines were toxic to XOR-deficient strains. In addition a range of AO substrates including N-heterocycles and aldehydes were significantly more toxic to *ma-l* AO-null strains. This study therefore provides definitive proof that both AO and XOR are involved in detoxification.

Investigations to determine the effect of MFE deficiencies on lifespan revealed that *ry* and *ma-l* strains had significantly reduced lifespan when compared with the wild type strain with the mean lifespan being reduced approximately 60% and 30% respectively

in these strains. The effect the MFE deficiencies have on heat and cold stress indicated that *ry* and *ma-l* strains had significantly reduced ability to survive, with the survivorship of the AO-null *ma-l* strain being most compromised in these conditions. The effect of the toxic chemicals, lifespan and temperature stress were rescued in *ry* mutant animals that had the normal *ry* (XOR) gene reinserted by transgenesis.

Cloning and DNA sequencing of the MCS gene in the *ma-l* strains revealed that the *mal-1* and *mal-fl* strains had a 6 bp insertion and a 23 bp deletion in exon 4 respectively that are predicted to lead to alterations of the deduced MCS protein structure, thus explaining the dual AO/XOR deficiency in these strains.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor Dr. Dougie Clarke for his advice, support and encouragement throughout the course of this research study and thesis write up. I acknowledge the Libyan government for financial support funding this study.

I acknowledge the University of Huddersfield for giving me the opportunity to complete this thesis.

I would like to thank my parents for their constant support and encouragement throughout this study.

I would like to thank to my wife and my daughters and son for their tolerance of my long hour absence from home in many evening, weekends and holidays during the past 4 years and for their endurance of the hardship in daily life associated with my studentship.

Finally, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Thank you and God bless all of you!

Table of Contents:

List of Tables:	ix
List of Figures:	xi
1. Introduction.....	1
1.2. Substrate specificity of aldehyde oxidase and xanthine oxidoreductase.	1
1.3. Molybdo-flavoenzyme structure	14
1.4. Molybdenum cofactor and the catalytic mechanism of molybdo-flavoenzymes.	16
1.5. Species variation in molybdo-flavoenzyme activity.....	24
1.5.1. Vertebrate molybdo-flavoenzymes	24
1.5.2. Insect molybdo-flavoenzymes	27
1.6. Molybdenum cofactor biosynthesis and molybdenum cofactor sulfuration.....	30
1.6.1. Identification of the molybdenum cofactor sulfurase gene and protein.	32
1.7. Molybdo-flavoenzyme deficiencies.....	36
1.7.1. Hereditary xanthinuria.	36
1.8. Molybdo-flavoenzyme deficiencies in insects.....	40
1.9. Effects of molybdo-flavoenzymes deficiencies on oxidative stress and xenobiotic toxicity in <i>D. melanogaster</i>	42
1.9.1. Effect of molybdo-flavoenzymes deficiencies on oxidative stress and xenobiotic toxicity in <i>D. melanogaster</i>	42
1.9.2. Effects of molybdo-flavoenzymes deficiencies on longevity of <i>D. melanogaster</i>	43
1.9.3. Effect of purine on molybdo-flavoenzymes deficiencies on <i>D. melanogaster</i>	44
1.10. Effect of molybdo-flavoenzymes deficiencies on temperature stress on <i>D. melanogaster</i>	44
1.11. Aims of the project.....	46
2.0. Materials and methods.	47
2.1. Animal studies.	47
2.1.1. <i>Drosophila melanogaster</i> strains used for research.	47
2.1.2. <i>Drosophila melanogaster</i> media.....	47
2.1.3. Procedure for transferring <i>Drosophila melanogaster</i> to new media bottles and vials.	49
2.2. <i>Drosophila melanogaster</i> lifespan tests.....	50
2.3. Heat and cold shock experiments.	51
2.4. <i>Drosophila melanogaster</i> toxicity tests.	51
2.4.1. Survival rate.	53

2.5. Analysis of the <i>in vivo</i> metabolism of molybdo-flavoenzyme substrates by <i>Drosophila melanogaster</i>	53
2.5.1. HPLC analysis of the xenobiotic substrates and metabolites.	54
2.5.2. Analytical procedure.....	55
2.5.2.1. Preparation of standards of calibration curves.....	55
2.5.2.2. Validation.....	55
2.5.2.2.1. Methods accuracy and precision.	55
2.5.2.3. Detection and Quantification limits.	56
2.5.3. HPLC assay of the purine based, N-heterocyclic and aldehydes substrates.....	56
2.5.4. <i>In vitro</i> analysis of <i>Drosophila melanogaster</i> molybdo-flavoenzymes activity.....	62
2.5.4.1. Preparation of cytosol from <i>Drosophila</i>	62
2.5.4.2. Gel filtration of <i>Drosophila</i> cytosol.....	62
2.5.4.3. Assessment of <i>in vitro</i> biotransformation of the purines, non-purines, N-heterocyclic and aldehydes substrates by <i>Drosophila</i> cytosol.....	63
2.6. Protein determination.....	65
2.6.1. Bicinchoninic acid reagents.	65
2.6.2. Bicinchoninic acid methodology.	65
2.7. Spectrophotometric determination of molybdo-flavoenzymes activity.....	66
2.7.1. Spectrophotometric determination of dimethylaminocinnamaldehyde oxidase activity.....	66
2.7.2. Spectrophotometric determination of phenanthridine oxidase activity.	67
2.7.3. Spectrophotometric determination of vanillin oxidase activity.	67
2.7.4. Spectrophotometric determination of xanthine oxidoreductase (XOR) activity.	68
2.7.5. Determination of specific activity.....	68
2.8. Determination of optimum conditions of molybdo-flavo enzymes.....	69
2.8.1. Determination of optimum pH of buffer.....	69
2.8.2. Temperature dependence of AO and XDH activities in <i>Drosophila</i> extracts.	69
2.8.3. Determination kinetic parameters (K_m) and maximum velocity (V_{max}) for molybdo-flavo enzymes enzyme at different substrate concentrations.	69
2.9. DNA analysis	71
2.9.1 Bioinformatics.....	71
2.9.2. Extraction of DNA from <i>Drosophila melanogaster</i>	71
2.9.3. Polymerase chain reaction cloning of molybdenum cofactor sulfurase gene.....	72
2.9.4. Polymerase chain reaction.	72

2.9.5. The PCR protocol.	73
2.9.6. Agarose gel electrophoresis.	74
2.9.7. Preparation of PCR samples for sequencing.	75
2.10. Statistical analysis.	76
2.10.1. Chi ² Statistical Tests.	76
2.10.2. T-test two tailed.	76
3. Results.	77
3.1. Quantification of molybdo-flavoenzyme activity in wild type and mutant <i>Drosophila melanogaster</i> strains.	77
3.1.1. Investigation of molybdo-flavoenzyme activities in Canton-S <i>Drosophila</i> strains using spectrophotometric assays.	77
3.1.2. Effect of pH and temperature on <i>Drosophila</i> molybdo-flavoenzymes activity.	80
3.1.3. Comparison of MFE activity in wild type and mutant <i>Drosophila melanogaster</i> strains.	81
3.1.4. Spectrophotometric determination of the kinetic parameters for aldehyde oxidase and xanthine oxidoreductase in <i>Drosophila</i> strains.	84
3.2. Quantification of molybdo-flavoenzyme activities in wild type and mutant <i>Drosophila melanogaster</i> strains by HPLC assays.	89
3.2.1. Validation.	89
3.2.1.1. Methods accuracy and precision of HPLC assays.	89
3.2.1.2. Detection and quantification limits.	90
3.2.2. HPLC analysis of the oxidation of purine based substrates by <i>Drosophila melanogaster</i> strains.	90
3.2.3. HPLC analysis of <i>D. melanogaster</i> strains with non purine and N-heterocyclic compounds.	98
3.2.4. HPLC <i>in vitro</i> analysis of <i>D. melanogaster</i> strains with endogenous vitamins and vitamin metabolites.	103
3.3. Investigation of the chemoprotective role of molybdo-flavoenzymes against xenobiotic toxicity.	107
3.4.1. Endogenous purine content of <i>Drosophila melanogaster</i> strains.	107
3.3.2. Investigation of the chemoprotective role of molybdo-flavoenzymes with purine and purine based drugs in <i>D. melanogaster</i> strains.	109
3.3.3. Investigation of the chemoprotective role of molybdo-flavoenzymes with toxicity of non-purine based N-heterocyclic compounds in <i>D. melanogaster</i> strains.	116
3.3.4. Investigation of the chemoprotective role of molybdo-flavoenzymes with vanillin and N ¹ -methylnicotinamide toxicity in <i>D. melanogaster</i> strains.	120

3.4. Lifespan Studies of molybdo-flavoenzymes-active and molybdo-flavoenzymes-deficient <i>Drosophila</i> strains.	126
3.5. Effect of heat and cold shock on <i>Drosophila melanogaster</i> strains.	128
3.5.1. Effect of 25, 37 and 39°C on <i>Drosophila melanogaster</i> strains.	128
3.5.2. Effect of 0 and -3 °C cold shock on <i>Drosophila melanogaster</i> strains.	130
3.6. Bioinformatics analysis of <i>Drosophila</i> molybdenum cofactor sulphurase (MCS) gene.	132
3.6.1. Design of PCR primers for the amplification of the <i>Drosophila</i> molybdenum cofactor sulphurase gene.	134
3.6.2. Optimisation of the PCR conditions for amplification of <i>Drosophila</i> molybdenum cofactor sulphurase gene.	134
3.6.3. Identification of the mutations responsible for molybdenum cofactor sulfurase deficiencies in <i>Drosophila melanogaster</i>	140
3.6.3.1. Identification of the mutations responsible for the MFE-deficiency observed in the <i>mal-1</i> <i>Drosophila</i> strain.	140
3.6.3.2. Identification of the mutations responsible for the MFE-deficiency observed in the <i>mal-fl</i> <i>Drosophila</i> strain.	142
4. Discussion.	144
4.1.1. Spectrophotometric and HPLC investigation of molybdo-flavoenzymes characterisation in wild type Canton-S <i>Drosophila</i> strain.	144
4.1.2. Spectrophotometric and HPLC investigation of molybdo-flavoenzyme characterisation in transgenic and molybdo-flavoenzyme deficient <i>Drosophila</i> strains. ...	148
4.1.3. <i>In vitro</i> Quantification of molybdo-flavoenzyme activities in wild type, transgenic and mutant <i>Drosophila melanogaster</i> strains by HPLC assays.	149
4.1.3.1. Biotransformation of xanthine and methylxanthines by <i>D. melanogaster</i> strains	150
4.1.3.2. Biotransformation of 6-mercaptopurine and methotrexate by <i>D. melanogaster</i> strains	151
4.1.3.3. Biotransformation of allopurinol by <i>D. melanogaster</i> strains	152
4.1.3.4. Biotransformation of pyrazinamide by <i>D. melanogaster</i> strains	153
4.1.3.5. Biotransformation of cinchonine by <i>D. melanogaster</i> strains	154
4.1.3.6. Biotransformation of vitamin B6 (pyridoxal) in <i>D. melanogaster</i> strains.....	155
4.1.3.7. Biotransformation of vitamin A (all- <i>trans</i> -retinaldehyde) in <i>D. melanogaster</i> strains	156
4.1.3.7. Biotransformation of N ¹ -methylnicotinamide by <i>D. melanogaster</i> strains	158
4.2. The chemoprotective role of molybdo-flavoenzymes against xenobiotic toxicity.	160
4.2.1. <i>In vivo</i> metabolism and toxicity of methylxanthines on <i>D. melanogaster</i> strains ...	160

4.2.2. <i>In vivo</i> biotransformation and toxicity of allopurinol on <i>D. melanogaster</i> strains..	164
4.2.3. <i>In vivo</i> biotransformation and toxicity of 6-mercaptopurine on <i>D. melanogaster</i> strains	165
4.2.4. <i>In vivo</i> biotransformation and toxicity of cinchonine on <i>D. melanogaster</i> strains..	166
4.2.5. <i>In vivo</i> biotransformation and toxicity of phenanthridine on <i>D. melanogaster</i> strains	166
4.2.6. <i>In vivo</i> biotransformation and toxicity of pyrazinamide on <i>D. melanogaster</i> strains	167
4.2.7. <i>In vivo</i> biotransformation and toxicity of N ¹ -methylnicotinamide on <i>D. melanogaster</i> strains	169
4.2.8. <i>In vivo</i> biotransformation and toxicity of vanillin on <i>D. melanogaster</i> strains	169
4.2.9. Summary of enzyme assays, <i>in vivo</i> metabolism and toxicity of compounds in <i>Drosophila</i> strains.	170
4.3. The effect of molybdo-flavoenzyme deficiencies on lifespan of <i>Drosophila melanogaster</i>	173
4.4. The effect of heat and cold shock on wild type and MFE- deficient <i>Drosophila</i> strains.	176
4.4.1. Effect of 25, 37 and 39 °C heat shock on <i>Drosophila melanogaster</i> strains.	176
4.4.2. Effect of 0 and -3 °C cold shock on <i>Drosophila melanogaster</i> strains.	178
4.5. Identification of mutations in the MCS Gene in <i>ma-l</i> mutants.	181
4.5.1. Cloning and sequencing of the <i>mal-1</i> mutant strains.	181
4.5.2. Cloning and sequencing of the <i>mal-fl</i> mutant strains.	184
4.6. Conclusions.	187
4.7. Recommendations for future work.	189
5. References	191
6.0. Appendices	221
6.1. Appendix 1	221
6.2. Appendix 2	222
6.3. Appendix 3	223
6.4. Appendix 4	232

List of Tables:

Table 1: Examples of mammalian molybdo-flavo enzymes substrates.....	12
Table 2: Reduction of various compounds by aldehyde oxidase.....	13
Table 3: Xanthine oxidoreductase catalyzed reduction of various compounds.....	14
Table 4: Summary of the substrate specificity and tissue distribution of molybdo-flavoenzymes in <i>Mus musculus</i>	26
Table 5: Summary of molybdo-flavoenzymes in non-rodent mammalian species.....	27
Table 6: Distribution of aldehyde oxidase and pyridoxal oxidase in adult <i>Drosophila melanogaster</i> tissues. + present and - : absent. Based on Cypher <i>et al.</i> , 1982.	28
Table 7: Summary of known AO and XOR genes and substrates in insects. * Substrates for individual enzymes not known	29
Table 8: Genetic deficiency of mammalian molybdo-flavoenzymes.	38
Table 9: Genetic deficiency of non- mammalian molybdo-flavoenzymes.	39
Table 10: Chemicals used for preparing agar medium (Ashburner, 1989).....	48
Table 11: A list of the xenobiotics used in toxicity experiments. Comments and appropriate solvents are indicated.....	53
Table 12: Summary of <i>in vivo</i> and <i>in vitro</i> HPLC analysis of purines, purines analog and N-heterocyclic substrates and products.....	58
Table 13: Summary of <i>in vivo</i> and <i>in vitro</i> HPLC analysis of N-heterocyclic and aldehyde substrates and products.....	59
Table 14: Summary of <i>in vitro</i> HPLC assays with molybdo-flavoenzyme substrates.....	64
Table 15: Dilution of BSA stock solution to give the required concentration to be used for the protein calibration standards.	65
Table 16: Dimethylaminocinnamaldehyde oxidase assay.	67
Table 17: Phenanthridine oxidase assay.	67
Table 18: Vanillin oxidase assay.	67
Table 19: The assay set used for XOR activity in <i>D. melanogaster</i>	68
Table 20: Summary of the primers designed for the PCR cloning of the <i>Drosophila</i> molybdenum cofactor sulfuryase gene. Nucleotide sequences and localization of the primers designed for the PCR amplification of <i>Drosophila</i> MCS gene.	73
Table 21: Summary of the volumes and concentrations of each component used in PCR reaction of the molybdenum sulfuryase cofactor gene.....	73
Table 22: Dimethylaminocinnamaldehyde, phenanthridine, vanillin and xanthine oxidase activities in <i>Drosophila</i> cytosol of different strains.	82
Table 23: Kinetic constants for molybdo-flavoenzyme substrates by <i>Drosophila</i> strains cytosol.	88
Table 24: Allopurinol oxidase activity in cytosol of <i>Drosophila melanogaster</i> strains.....	98
Table 25: Pyrazinamide and cinchonine oxidase activity in cytosol of <i>Drosophila melanogaster</i> strains.	103

Table 26: The oxidase activity towards pyridoxal, <i>all-trans</i> retinal and N ¹ -methylnicotinamide by cytosol of <i>Drosophila</i> strains.	106
Table 27: Xanthine, hypoxanthine and uric acid content of wild type, transgenic and mutant <i>D. melanogaster</i> strains.	109
Table 28: Quantification of allopurinol and oxipurinol in fly extracts after 4 days of 20 mM allopurinol treatment.	116
Table 29: Quantification of phenanthridine and 6(5H)-phenanthridone in fly extract after 4 days of 20 mM phenanthridine treatment.	118
Table 30: Statistical difference of mean lifespan between 20 mM vanillin treated and untreated <i>Drosophila</i> strains.	122
Table 31: Quantification of vanillin and vanillic acid in fly extract after 4 days of 20 mM vanillin treatment.	125
Table 32: Mean lifespan and maximum life of wild type, transgenic and mutant strains.	127
Table 33: Statistical difference in survival rate between wild type and mutant strains after exposure to the heat shock at 37 and 39°C.	129
Table 34: Statistical difference in survival rate between wild type and mutant strains after exposure to the cold shock at 0 and -3°C.	131
Table 35: Summary of the experimental conditions and outcomes of the PCR of the <i>Drosophila</i> MCS gene.	135
Table 36: Summary of the <i>in vitro</i> oxidation of molybdoflavoenzyme substrates by <i>Drosophila</i> strains.	159
Table 37: Summary of the toxicity, <i>in vivo</i> and <i>in vitro</i> oxidation of purines, non purines, N-heterocyclic and aldehyde substrates by <i>Drosophila</i> strains used in this study.	171
Table 38: Summary of the toxicity of purines based, purines analog, N-heterocycles and aldehyde compounds to <i>Drosophila melanogaster</i> strains.	172

List of Figures:

Figure 1: Role of xanthine oxidoreductase in endogenous purine catabolism in mammals.	2
Figure 2: Role of xanthine oxidoreductase in caffeine metabolism. CYP: Cytochrome P-450. XOR: Xanthine oxidoreductase.	4
Figure 3: Metabolism of phthalazine in mammals by aldehyde oxidase to 1-phthalazinone (Beedham <i>et al.</i> , 1987).	5
Figure 4: Metabolism of vitamin B3 in mammals.	6
Figure 5: Conversion of retinal (vitamin A) to its corresponding carboxylic acid (retinoic acid) and oxidation of vitamin B6 (pyridoxal) to its corresponding carboxylic acid (4-pyridoxic acid) catalyzed by aldehyde oxidase. Based on Macrae <i>et al.</i> , 1984; Tomita <i>et al.</i> , 1993.	8
Figure 6: Oxidation of phenanthridine to 6(5H)-phenanthridone and oxidation of methotrexate to 7-hydroxymethotrexate by AO. Based on Beedham, 2001; Kitamura <i>et al.</i> , 2006; Lavoie <i>et al.</i> , 1985; Sugihara <i>et al.</i> , 1999.	9
Figure 7: Conversion of vanillin to its corresponding carboxylic acid catalyzed by aldehyde oxidase. Based on Panoutsopoulos <i>et al.</i> , 2004; Rashidi, 1996.	10
Figure 8: The oxidation of 6-mercaptopurine, a common substrate for aldehyde oxidase and xanthine oxidoreductase to different oxidative products. Based on (Beedham, 2001; Kitamura <i>et al.</i> , 2006; Rashidi, 1996; Rashidi <i>et al.</i> , 2007; Zimm <i>et al.</i> , 1984).	11
Figure 9: Schematic diagram of the domain structure of the molybdenum centre in enzymes of the XDH/AO enzyme family.	15
Figure 10: Crystal structures of bovine milk XOR dimer with the three domains represented in different colours. Iron sulfur-center domain (residues 3–165; red), FAD domain (residues 226–531; green) and Mo-pterin cofactor domain (residues 590–1331; blue) (Enroth <i>et al.</i> , 2000). The active site occurs between the two Lys569 and Lys551 residues. Used with permission from Professor Takeshi Nishino.	16
Figure 11: Molybdenum cofactor. The active site structure is attached to the pyranopterin compound by way of two enedithiolate side chains (red colour) to produce the molybdenum cofactor (Brondino <i>et al.</i> , 2005).	17
Figure 12: Proposed sequence of electron flow out of the molybdenum centre of molybdo-flavoenzymes. Based on Hille & Nishino, 1995; Nishino & Okamoto, 2000.	17
Figure 13: Oxygen atom transfer reaction by molybdo-flavoenzymes.	18
Figure 14: Oxygen atom transfer reaction molybdo-flavoenzyme involving an aldehyde and xanthine. Based on Hille & Nishino, 1995; Okamoto <i>et al.</i> , 2004.	18
Figure 15: The reaction mechanism for XOR with xanthine as a substrate.	21
Figure 16: A general reaction mechanism of the AO enzyme with an aldehyde substrate.	22

Figure 17: Reduction pathways occurring in XOR/AO and XDH (Beedham, 1998; Hille, 1996).	23
Figure 18: Summary of molybdenum cofactor synthesis based on Mendel & Bittner, 2006.	31
Figure 19: Domain structure and function of Moco sulfurase <i>Aba3</i> . Based on Schwarz <i>et al.</i> , 2009, contains an N-terminal <i>NifS</i> -like domain that binds pyridoxal phosphate (PLP) via a conserved lysine residue and desulfurates L-cysteine to L-alanine, thereby generating a cysteine persulfide.	34
Figure 20: Wild type eye colour on the left and <i>ry</i> ⁵⁰⁶ mutant eye colour on the right.	41
Figure 21: Bottle container for <i>D. melanogaster</i> media.	49
Figure 22: Etherising container used for etherising flies.	50
Figure 23: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for purines.	60
Figure 24: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for allopurinol and oxipurinol.	60
Figure 25: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for N ¹ -methylnicotinamide.	61
Figure 26: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for phenanthridine and 6(5H)-phenanthridone.	61
Figure 27: A plot of the Eadie- Hofstee.	70
Figure 28: Spectrophotometric assay of aldehyde oxidase and xanthine dehydrogenase activities using Canton-S strain cytosol at 37°C with AO and XDH substrates.	79
Figure 29: Effect of the assay incubation temperature on the activity of Canton-S strain AO and XDH.	80
Figure 30: Effect of the pH on the activity of Canton-S strain AO (blue line) and XDH (red line).	81
Figure 31: HPLC analysis of the <i>in vitro</i> biotransformation of vanillin and phenanthridine by Canton-S, rosy and maroon-like strains cytosol.	83
Figure 32: HPLC analysis of the <i>in vitro</i> biotransformation of xanthine by Canton-S, rosy and maroon-like cytosol after 120 minutes incubation time at 37°C.	84
Figure 33: Eadie- Hofstee plot of AO and XDH activities in Canton-S <i>Drosophila</i> strain.	86
Figure 34: Eadie- Hofstee plot of AO activity in <i>ry</i> ⁵⁰⁶ <i>Drosophila</i> strain.	87
Figure 35: HPLC analysis of the <i>in vitro</i> biotransformation of xanthine by Canton-S cytosol.	91
Figure 36: HPLC analysis of the <i>in vitro</i> biotransformation of 1-methylxanthine (1-MX) by Canton-S cytosol at 37°C.	92
Figure 37: HPLC analysis of analytes produced following the <i>in vitro</i> incubation of 6-mercaptopurine with Canton-S cytosol with and without NAD ⁺ at 37°C.	94

Figure 38: HPLC analysis of the <i>in vitro</i> biotransformation of 6-mercaptopurine by rabbit liver cytosol at 0 and 60 minutes.	95
Figure 39: HPLC analysis of the <i>in vitro</i> biotransformation of allopurinol by Canton-S cytosol strain at 37°C with and without NAD ⁺	96
Figure 40: HPLC analysis of the <i>in vitro</i> biotransformation of allopurinol by <i>ry</i> ⁵⁰⁶ cytosol strain.	97
Figure 41: HPLC analysis of the <i>in vitro</i> biotransformation of pyrazinamide by Canton-S cytosol strain at 37°C with and without NAD ⁺	99
Figure 42: HPLC analysis of the <i>in vitro</i> biotransformation of pyrazinamide by <i>ry</i> ⁵⁰⁶ <i>Drosophila melanogaster</i> cytosol at 37°C.	100
Figure 43: HPLC analysis of the <i>in vitro</i> biotransformation of cinchonine to 2'-cinchoninone by rosy cytosol.	101
Figure 44: HPLC analysis of analytes produced following the <i>in vitro</i> incubation of methotrexate with <i>Drosophila</i> and rabbit liver cytosol.	102
Figure 45: HPLC analysis of the <i>in vitro</i> biotransformation of endogenous compounds (pyridoxal and <i>all-trans</i> retinal) by Canton-S cytosol.	104
Figure 46: HPLC analysis of the <i>in vitro</i> biotransformation of N ¹ -methylnicotinamide by Canton-S cytosol.	105
Figure 47: HPLC analysis of endogenous purines in untreated <i>Drosophila melanogaster</i> strains.	108
Figure 48: Toxicity of methyl-xanthines to <i>Drosophila melanogaster</i> strains (a) caffeine (20 mM), (b) theobromine (20mM), (c) theophylline (20 mM) and (d) paraxanthine (100 mM).	110
Figure 49: HPLC analysis wild type Canton-S <i>Drosophila melanogaster</i> extracts after 4 days treatment with caffeine.	111
Figure 50: HPLC chromatogram of purines found in Canton-S <i>Drosophila</i> after 4 days of treatment with paraxanthine, theobromine and theophylline.	112
Figure 51: Toxicity of 6-mercaptopurine and allopurinol to wild type, transgenic and mutant of <i>Drosophila melanogaster</i> strains.	114
Figure 52: HPLC analysis of allopurinol metabolism in <i>Drosophila melanogaster</i> strains.	115
Figure 53: Toxicity of 20 mM phenanthridine, pyrazinamide and cinchonine to wild type, transgenic and mutant of <i>Drosophila melanogaster</i> strains.	117
Figure 54: HPLC analysis of phenanthridine and cinchonine biotransformation in Canton-S <i>Drosophila melanogaster</i> strain.	119
Figure 55: Toxicity of 20 mM N ¹ -methylnicotinamide to wild type, transgenic and mutant of <i>Drosophila melanogaster</i> strains.	120
Figure 56: Toxicity of 20 mM vanillin to wild type, transgenic and mutant of <i>Drosophila melanogaster</i> strains.	121
Figure 57: Comparison of the effect of 20 mM vanillin on lifespan of <i>Drosophila melanogaster</i> strains.	122

Figure 58: Toxicity of 100 mM vanillin to wild type, transgenic and mutant <i>Drosophila melanogaster</i> strains.	123
Figure 59: HPLC analysis of vanillin biotransformation in <i>Drosophila melanogaster</i> strains.	124
Figure 60: The lifespan of Canton-S, transgenic, <i>ry</i> ⁵⁰⁶ , <i>mal-1</i> and <i>mal-f1</i> strains.	126
Figure 61: The survival percentage of <i>Drosophila</i> strains exposed to heat shock for different durations at 37 and 39°C temperature.	129
Figure 62: The survival percentage of <i>Drosophila</i> strains exposed to cold shock for different durations at 0 and -3°C.	131
Figure 63: Complete gene sequence for the MCS gene in <i>Drosophila melanogaster</i> showing deduced amino acid sequence, exonic sequence shown in upper case black letters and intronic sequence shown in lower case black letters.	133
Figure 64: Agarose gel electrophoresis of products obtained by PCR amplifying different regions of Canton-S <i>Drosophila</i> molybdenum cofactor sulfurase gene. DMSC1-9 sets used from Table 27.	136
Figure 65: Comparison of the nucleotide sequence of the Canton-S <i>D. melanogaster</i> strain MCS gene (CSDMCS) obtained in this study with those on GeneBank. ..	137
Figure 66: Comparison of the DNA sequencing electrophoretograms obtained from the wild type and the <i>mal-1</i> <i>D. melanogaster</i> strains using primer set DMCS-8.	140
Figure 67: Comparison of the <i>mal-1</i> with Canton-S wild type molybdenum cofactor sulfurase nucleotide and deduced protein sequences at the site of the mutation.	141
Figure 68: Comparison of the sequencing electrophoretograms of the MCS gene from the wild type and the <i>mal-f1</i> <i>D. melanogaster</i> strains using primer set DMCS-8.	142
Figure 69: Nucleotide sequence alignment of the Canton-S and <i>mal-f1</i> molybdenum cofactor sulfurase gene at the site of the 23bp deletion in exon 4.	143
Figure 70: Comparison of the predicted molybdenum cofactor sulfurase protein sequence of the <i>mal-f1</i> and Canton-S wild type strain. Sequences were aligned using CLUSTAL X program (Larkin <i>et al.</i> , 2007).	143
Figure 71: The principal metabolic pathways of pyrazinamide in mammals (Kitamura <i>et al.</i> , 2006; Lacroix <i>et al.</i> , 1989).	168
Figure 72: Alignment of MCS protein sequences from species throughout evolution.	183
Figure 73: Amino acid sequence alignment of deduced <i>ma-l</i> MCS proteins and homologs in other species.	185

List of Abbreviations:

Abbreviation: **Definition:**

17U:	1, 7-dimethyluric acid
1U:	1-methyluric acid
1X:	1-methylxanthine
2-PY:	N ¹ -methyl-2-pyridone-5-carboxamide
3X:	3-methylxanthine
4-PA:	4-Pyridoxic acid
4-PY:	N ¹ -methyl-4-pyridone-3-carboxamide
6-MP:	6-mercaptopurine
6-TX:	6-thioxanthine
7X:	7-methylxanthine
7-OH-MTX:	7-hydroxymethotrexate
ACN:	Acetonitrile
AD:	Adenosine deaminase
ADase:	Adenosine deaminase
ALDH:	Aldehyde dehydrogenase
AMP:	Guanosine monophosphate
AO:	Aldehyde oxidase
AOH1:	Aldehyde oxidase homologue 1
ATR:	All-trans retinal (vitamin A).
bp:	Base pair
BSA:	Bovine serum albumin
137X:	Caffeine
cDNA:	Complementary deoxyribonucleic acid
Cin:	Cinnamon
CV:	Coefficient of variation
CS:	Canton-S
CYP:	Cytochrome P450
<i>D. gigas</i>	<i>Desulfovibrio gigas</i>
Da:	Dalton

Abbreviation: **Definition:**

DAD:	Diode array detector
DMAC:	Dimethylaminocinnamaldehyde
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleotide triphosphate
EDTA:	Ethylenediamine tetra acetic acid.
FAD:	Flavin adenine dinucleotide
GD:	guanine deaminase
GDase:	Guanine deaminase
GMP:	Xanthosine monophosphate
GTP:	Guanine triphosphate
HMCS:	Human molybdenum cofactor sulphurase
HPLC:	High performance liquid chromatography
H ₂ O ₂ :	Hydrogen peroxide
kb:	Kilobase
K _m :	Michaelis constant
LC-MS:	Liquid chromatography MS
LOD:	Limit of detection
LOQ:	Lower limit of Quantification
Lxd:	Low xanthine dehydrogenase
<i>ma-l</i> :	Maroon-like
<i>Methuselah</i> :	<i>mtH</i>
MFes:	Molybdoflavoenzymes
NMN:	N ¹ -methylnicotinamide
MoCoD:	Molybdenum cofactor deficiency
MCS:	Molybdenum cofactor sulfurase
MgCl ₂ :	Magnesium chloride
mM:	Millimolar
MPT:	Molybdopterin
mRNA:	Messenger ribonucleic acid
MTX:	Methotrexate

Abbreviation: **Definition:**

Na ₂ HPO ₄ :	Disodium hydrogen phosphate
NAD ⁺ :	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National Centre for Biotechnology Information
17X:	Paraxanthine
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
PL:	Pyridoxal.
PNP:	Purine nucleoside phosphorylase
PO:	Pyridoxal oxidase
PZA:	Pyrazinamide
<i>R. capsulatus</i> :	<i>Rhodobacter capsulatus</i>
RA:	Retinoic acid (vitamin A acid).
RHPLC:	Reversed-phase liquid chromatography
Ry:	Rosy
SO:	Sulfite oxidase
Taq:	<i>Thermus aquaticus</i>
TBE:	Tris borate EDTA
TE:	Tris EDTA
RSD:	Relative standard deviation
37X:	Theobromine
13X:	Theophylline
U:	Uric acid
UGT:	UDP-glucuronosyltransferase
Ur:	Uracil
X:	Xanthine
XDH:	Xanthine dehydrogenase
XMP:	Inosine monophosphate
XO:	Xanthine oxidase.
XOR:	Xanthine oxidoreductase

1. Introduction.

Xenobiotics are compounds that are foreign to the body, which include drugs, pollutants and other substances that are not normally present in the body that are potentially toxic. Xenobiotic metabolism is the series of metabolic reactions that change the chemical structure of xenobiotics; generally acting to detoxify the toxic chemical compounds. Sometimes, however, the product of xenobiotic metabolism can be the cause of toxic effects (Hodgson & Smart, 2001). Of the biotransformations that occur in animals oxidation plays a major role in the metabolism of foreign compounds. Although the microsomal cytochrome P-450 mono-oxygenase system is of major importance in this respect, enzymes present in the cytosol also contribute to this process. This thesis is concerned with two of these enzymes aldehyde oxidase (AO; EC 1.2.3.1) and xanthine oxidoreductase (XOR; EC 1.2.3.2) that are molybdo-flavoenzymes found in nearly every organism from bacteria to human (Beedham, 2001; Garattini *et al.*, 2008, 2009; Garattini *et al.*, 2003; Garattini & Terao, 2011).

1.2. Substrate specificity of aldehyde oxidase and xanthine oxidoreductase.

AO and XOR catalyze the oxidation of many different N-heterocyclic compounds as well as aliphatic and aromatic aldehydes to their corresponding lactam and carboxylic acids respectively (Beedham, 2001; Garattini *et al.*, 2008; Garattini *et al.*, 2003; Garattini & Terao, 2011, 2012). Despite their ability to oxidize the same class of substrates and their structural similarities, AO and XOR vary in their substrate specificities and their response to inhibitors (Kundu *et al.*, 2007; Mendel & Bittner, 2006). For this reason, it is experimentally possible to differentiate between the enzymes by measuring the activities obtained by reacting them with different substrates or by subjecting the oxidation reactions to different inhibitors. For example, the well known endogenous substrate xanthine is converted to uric acid by XOR but

AO is incapable of catalyzing this reaction (Krenitsky *et al.*, 1986). The physiological role of XOR is mainly in purine catabolism in which it catalyzes the oxidation of hypoxanthine to xanthine and then to the more polar compound uric acid (Figure 1) (Garattini *et al.*, 2008; Kitamura *et al.*, 2006; Krenitsky *et al.*, 1972; Nishino & Okamoto, 2000).

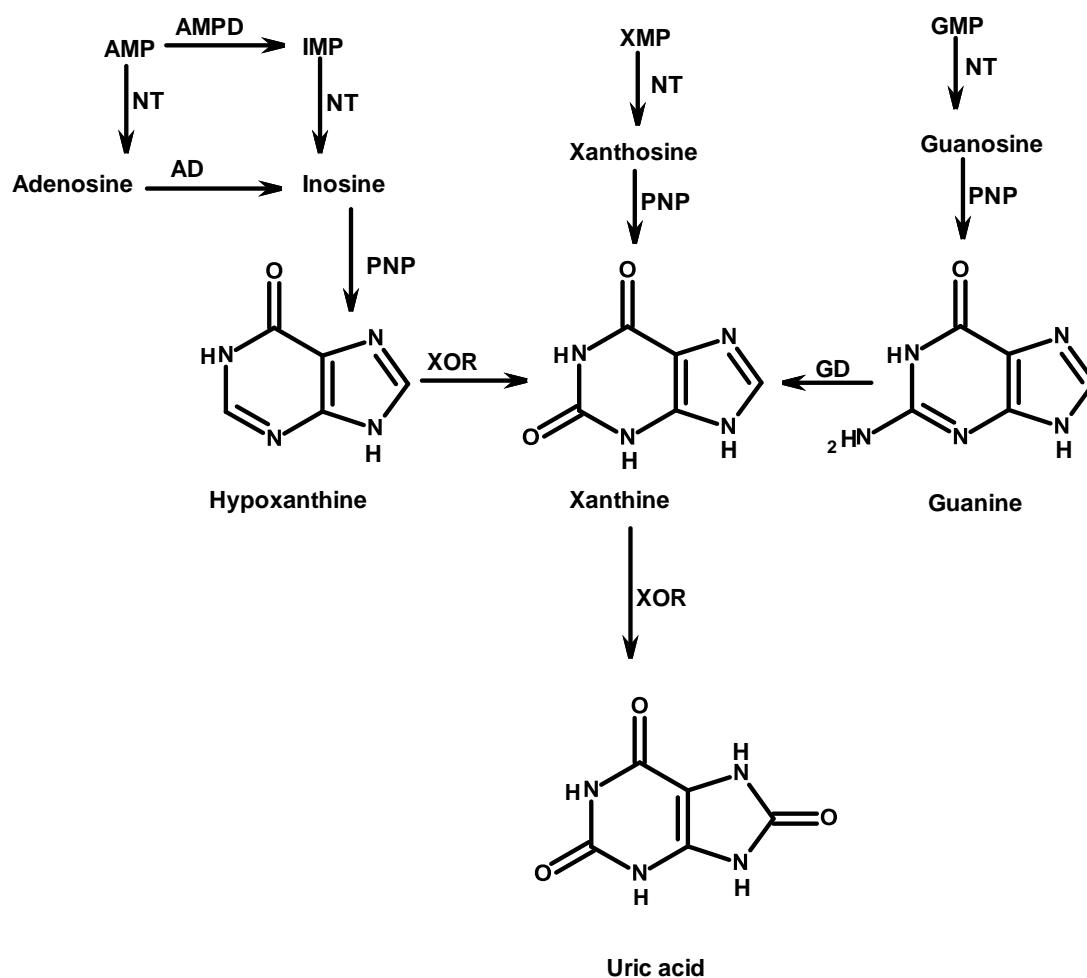


Figure 1: Role of xanthine oxidoreductase in endogenous purine catabolism in mammals.

AMP: Adenosine monophosphate, IMP: Inosine monophosphate, XMP: Xanthosine monophosphate, GMP: Guanosine monophosphate, AMPD: AMP deaminase, NT: nucleotidase, AD: adenosine deaminase, PNP: purine nucleoside phosphorylase, GD: guanine deaminase, XOR: xanthine oxidoreductase (Boueiz *et al.*, 2008).

Mammalian XOR can exist as a dehydrogenase (XDH) and oxidase (XO) form. XDH is the predominant form of the enzyme under non-pathological (normal) conditions (Borges *et al.*, 2002). The difference between the two forms is largely based on the electron acceptor utilized for catalytic activity. XO transfers reducing equivalents to O₂, whereas XDH transfers them predominantly to NAD⁺ and to a much lesser extent oxygen (Hille & Nishino, 1995; Nishino *et al.*, 2005). XDH can be converted irreversibly to XO by limited proteolysis or by reversible oxidation of cysteine residues (Nishino & Nishino, 1997; Nishino *et al.*, 2005).

Due to their structural similarity to the endogenous substrates many exogenous methylated xanthines are also substrates for XOR. Methylxanthines are generally demethylated by cytochrome P450 1A2 (CYP1A2) and the metabolites generated are metabolised by XOR (Arnaud, 2011; Hamelin *et al.*, 1994; Krenitsky *et al.*, 1972; Streetman *et al.*, 2000) (shown in figure 2). Bovine milk xanthine oxidase catalyses the oxidation of 7-methylxanthine, 1-methylxanthine and theophylline, to varying degrees with the 1-methylxanthine being the best substrate (Krenitsky *et al.*, 1972).

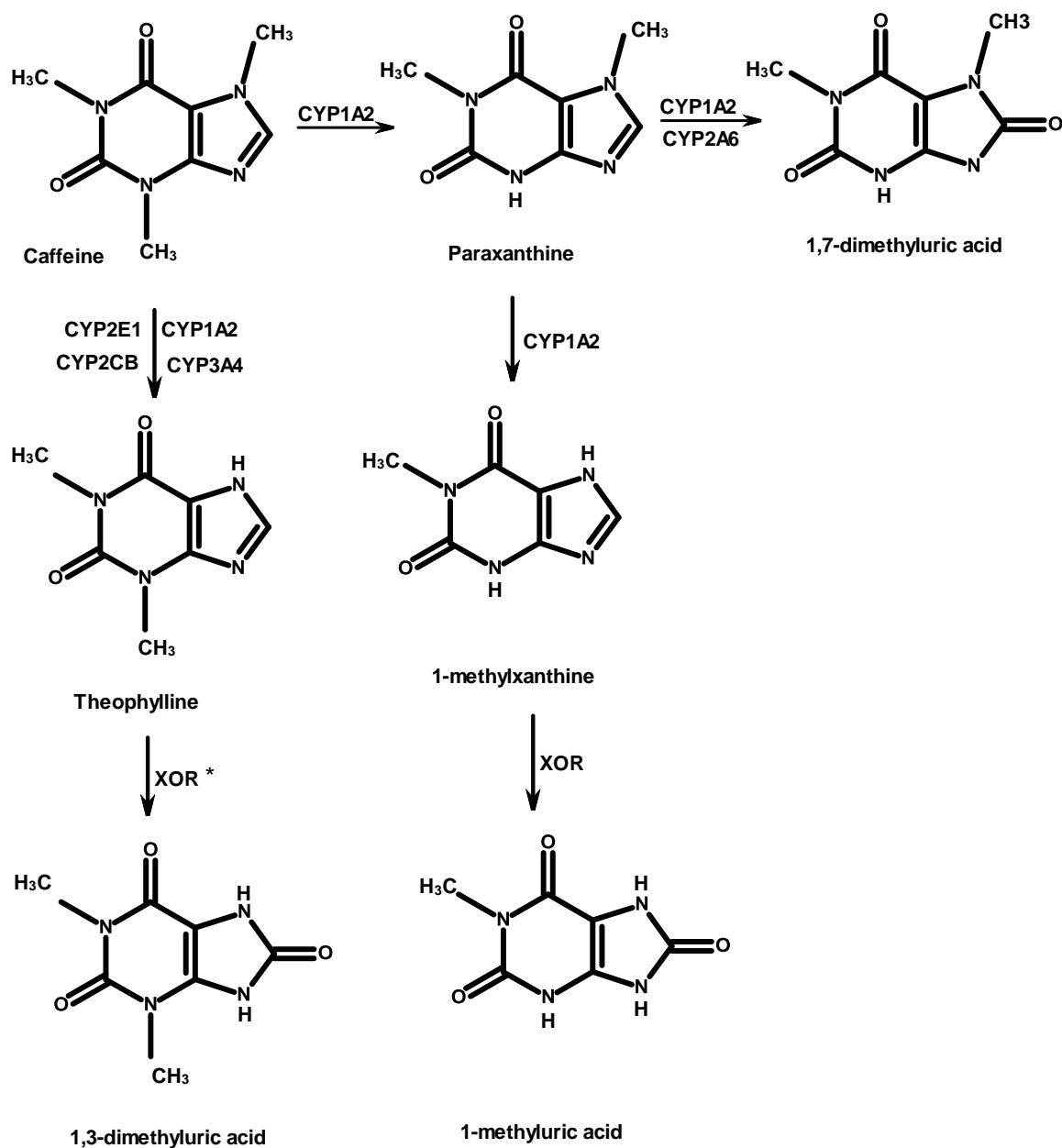


Figure 2: Role of xanthine oxidoreductase in caffeine metabolism. CYP: Cytochrome P-450. XOR: Xanthine oxidoreductase.

* indicates not unequivocally proven (Arnaud, 2011; Baselt & Cravey, 1996; Hamelin *et al.*, 1994; Relling *et al.*, 1992).

Despite its name, AO not only oxidizes aldehydes but also catalyzes the hydroxylation of aromatic azaheterocycles possessing a $-\text{CH}=\text{N}-$ moiety such as phthalazine (Figure 3) (Beedham *et al.*, 1990; Panoutsopoulos & Beedham, 2004; Stubley *et al.*, 1979) or

aromatic and non-aromatic charged heterocycles with a $-\text{CH}=\text{N}^+$ group like the vitamin B3 metabolite (N^1 -methylnicotinamide) (Figure 4) (Beedham, 1987, 2001; Kundu *et al.*, 2007; Shibata *et al.*, 1988; Sugihara *et al.*, 2006). AO catalyzes the oxidation of aldehydes and nitrogeneous heterocyclic xenobiotics like vanillin and phenanthridine as well as the oxidative metabolism of a variety of endogenous compounds like pyridoxal, retinaldehyde and N^1 -methylnicotinamide (Beedham, 1985, 1987; Beedham *et al.*, 1995; Egashira *et al.*, 1999; Huang *et al.*, 1999; Kitamura *et al.*, 2006; Rashidi *et al.*, 1997; Shibata *et al.*, 1988; Stanulovic & Chaykin, 1971). Although AO catalyses the biotransformation of several endogenous compounds, the absolute primary physiological function of AO is yet to be determined. The physiological importance of aldehyde oxidase's role in aldehyde oxidation is in question due to the fact that the Michaelis constant (K_m) for AO and XO is higher for aliphatic aldehydes than is that of another mammalian enzyme, aldehyde dehydrogenase (ALDH) [EC; 1.2.1.3] (Jakoby & Ziegler, 1990; Panoutsopoulos *et al.*, 2004).

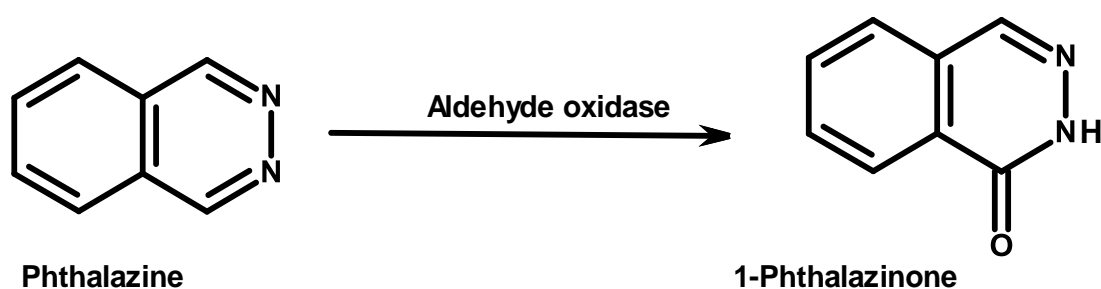


Figure 3: Metabolism of phthalazine in mammals by aldehyde oxidase to 1-phthalazinone (Beedham *et al.*, 1987).

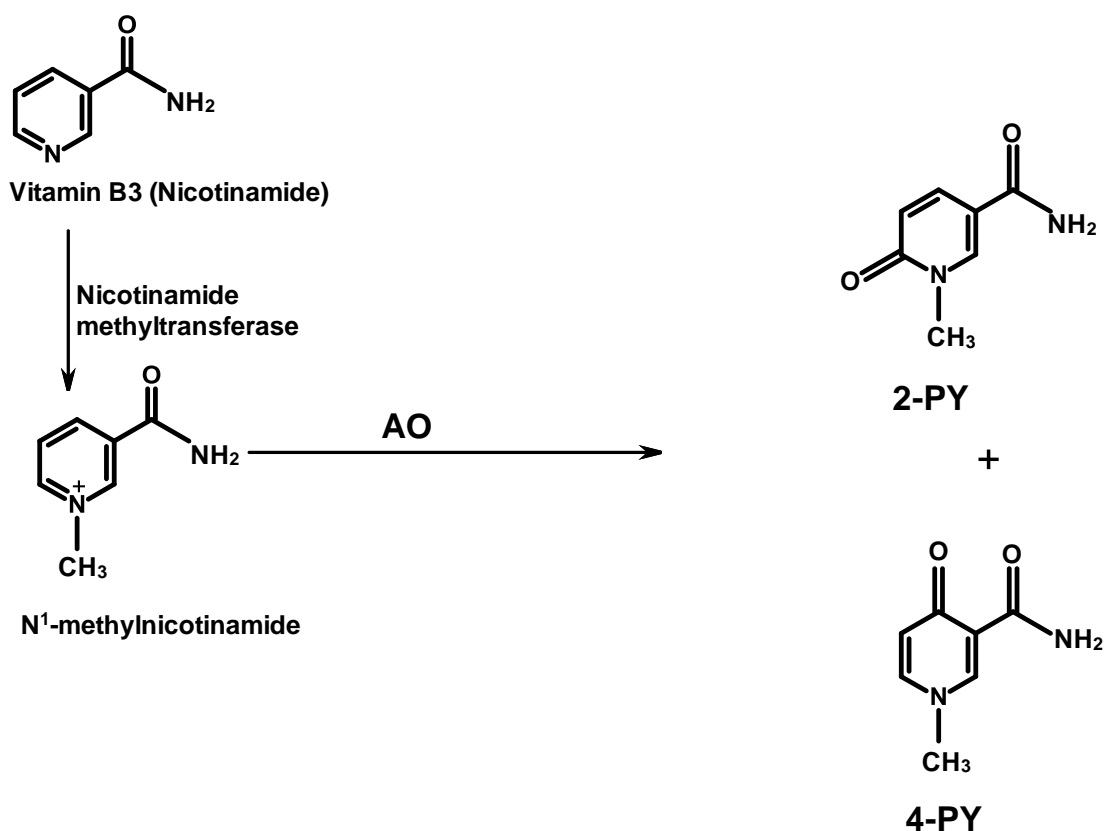


Figure 4: Metabolism of vitamin B3 in mammals.

Methylation of nicotinamide by nicotinamide methyltransferase followed by oxidation of N¹-methylnicotinamide by AO to N¹-methyl-2-pyridone-5-carboxamide (2-PY) and N¹-methyl-4-pyridone-5-carboxamide (4-PY). Based on Beedham, 2001; Egashira *et al.*, 1999; Shibata *et al.*, 1989 and 2006.

As well as N¹-methylnicotinamide there are other examples of endogenous compounds that are AO substrates (Figure 4). Two notable endogenous substrates for AO include retinaldehyde and pyridoxal (Beedham, 2001; Garattini *et al.*, 2008; Garattini *et al.*, 2003; Garattini & Terao, 2011, 2012; Huang *et al.*, 1999; Kitamura *et al.*, 2006). Retinaldehyde is the principle component of visual pigments and for this reason it has been suggested that aldehyde oxidase may play an important part of the overall visual process since it catalyzes the biotransformation of this aldehyde to its corresponding carboxylic acid, retinoic acid, which is the active form of vitamin A

(Calzei *et al.*, 1995; Garattini *et al.*, 2008; Garattini & Terao, 2011, 2012; Huang *et al.*, 1999; Stanulovic & Chaykin, 1971). The involvement of AO in all-*trans* retinaldehyde (RA) oxidation to all-*trans* retinoic acid (ATR) was first seen in rabbit liver cytosol, where it was observed that a fraction of the oxidizing activity did not require addition of NAD⁺ and was due to a molybdo-flavoenzymes (MFE) (Garattini *et al.*, 2008; Tomita *et al.*, 1993; Tsujita *et al.*, 1994). As well as its ability to catalyses the biotransformation of vitamin A and B3 metabolites. AO also converts vitamin B6 (pyridoxal) to 4-pyridoxic acid (Figure 5) (Tomita *et al.*, 1993). Pyridoxal is a good substrate of insect AO *in vitro* (Browder & Williamson, 1976; Cypher *et al.*, 1982). In mice Garattini *et al.* (2008) reported that pyridoxal can be oxidized by purified mouse aldehyde oxidase AOX1 and AOH1, although it is not an efficient substrate in the case of AOH2.

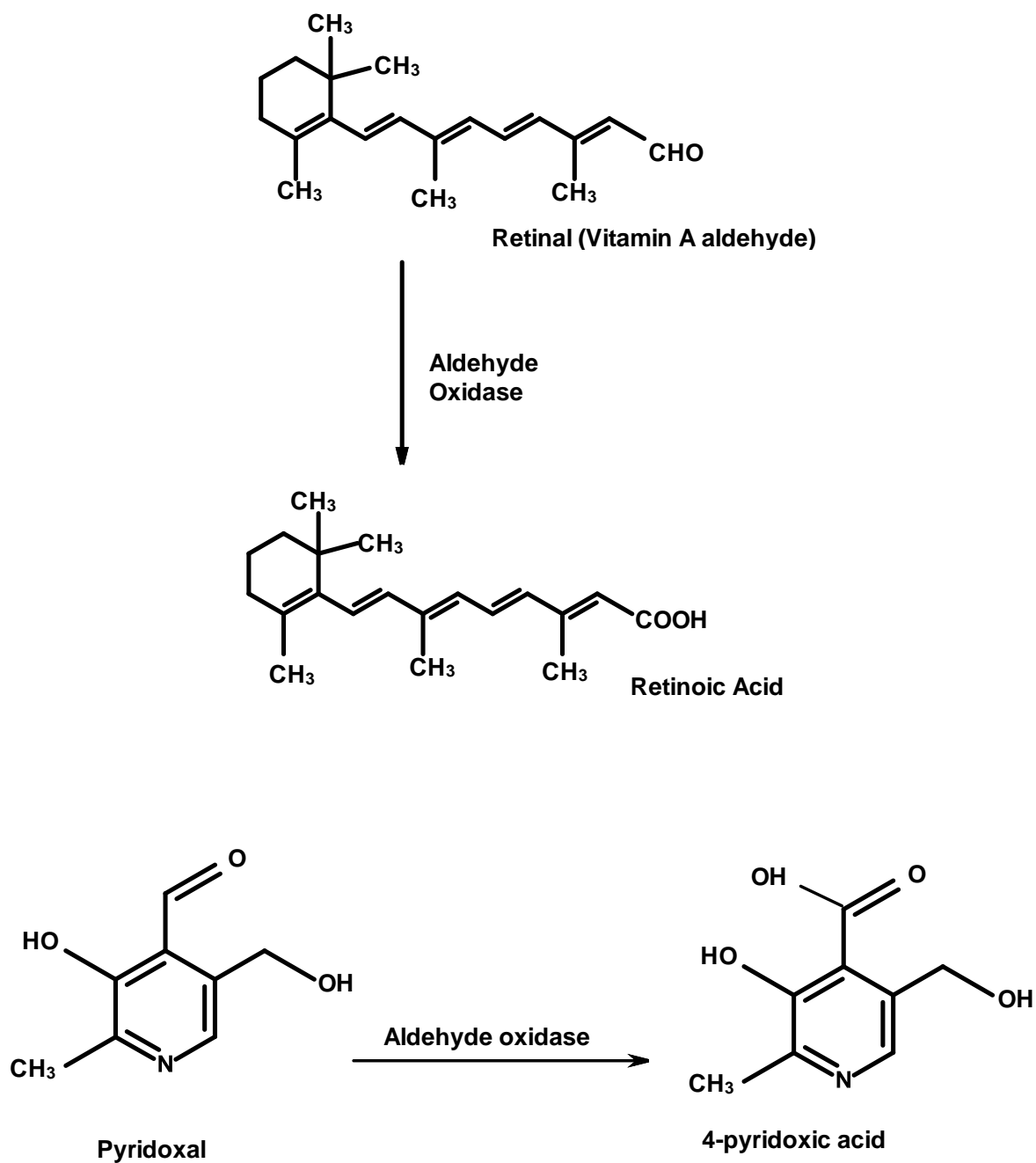


Figure 5: Conversion of retinal (vitamin A) to its corresponding carboxylic acid (retinoic acid) and oxidation of vitamin B6 (pyridoxal) to its corresponding carboxylic acid (4-pyridoxic acid) catalyzed by aldehyde oxidase. Based on Macrae *et al.*, 1984; Tomita *et al.*, 1993.

Examples of xenobiotics that are substrates for AO are shown in figure 6. Phenanthridine is a good example of an exogenous toxic pollutant, which is a prototypical uncharged xenobiotic substrate of AO. The oxidative product, 6(5H)-

6(5H)-phenanthridone is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Figure 6) (Beedham, 2001; Kitamura *et al.*, 2006; LaVoie *et al.*, 1985). The anti-leukemia drug methotrexate (4-amino-N10-methylpteroyl-L-glutamic acid) formally known as amethopterin, is a pteridine compound that is metabolised to its major metabolite 7-hydroxymethotrexate by mammalian liver aldehyde oxidase (Figure 6) (Garattini *et al.*, 2008; Garattini & Terao, 2011, 2012; Kitamura *et al.*, 1999; Kitamura *et al.*, 2006; Moriyasu *et al.*, 2006).

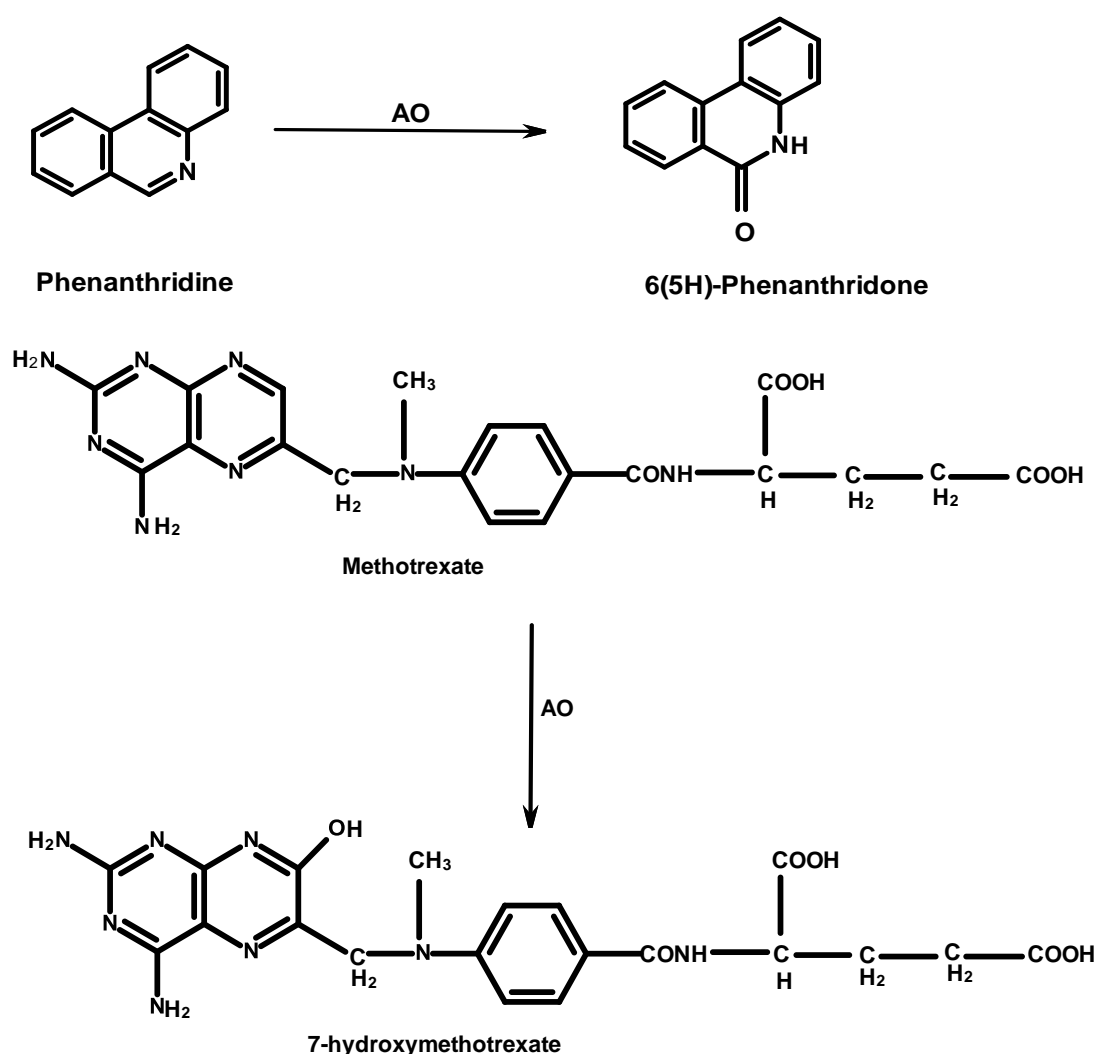


Figure 6: Oxidation of phenanthridine to 6(5H)-phenanthridone and oxidation of methotrexate to 7-hydroxymethotrexate by AO. Based on Beedham, 2001; Kitamura *et al.*, 2006; Lavoie *et al.*, 1985; Sugihara *et al.*, 1999.

Certain compounds are substrates for both AO and XOR two such compounds are vanillin and 6-mercaptopurine (Figures 7 and 8). Vanillin, found in food stuffs is an aromatic aldehyde compound that was rapidly converted to its metabolite vanillic acid by AO with negligible contribution from XO or aldehyde dehydrogenase (ALDH) (Figure 7) (Beedham, 2001; Panoutsopoulos & Beedham, 2004; Rashidi, 1996; Sahi *et al.*, 2008).

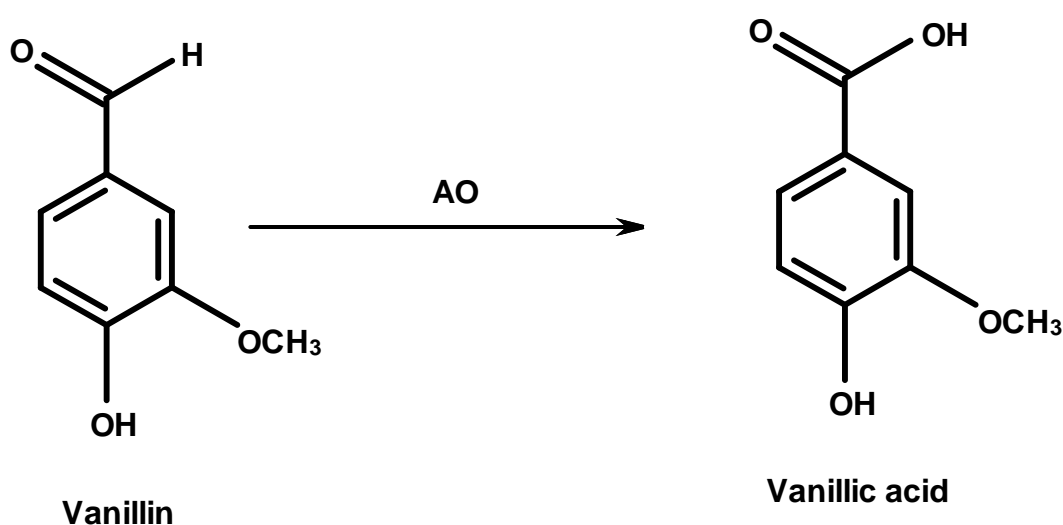


Figure 7: Conversion of vanillin to its corresponding carboxylic acid catalyzed by aldehyde oxidase. Based on Panoutsopoulos *et al.*, 2004; Rashidi, 1996.

AO like XO can also catalyze the oxidation of certain purines and in some cases with higher activity than XO (Beedham, 2001). An example is that 6-mercaptopurine is oxidized to 6-methylmercaptopurin-8-one by AO via methyltransferase enzyme and to 6-mercaptopurin-2-one (6-thioxanthine) by XOR and to 6-thiouric acid (end product) by both XOR and AO (Figure 8). This is according administration of 6-mercaptopurine drug (Beedham, 2001; Kitamura *et al.*, 2006; Rashidi *et al.*, 2007).

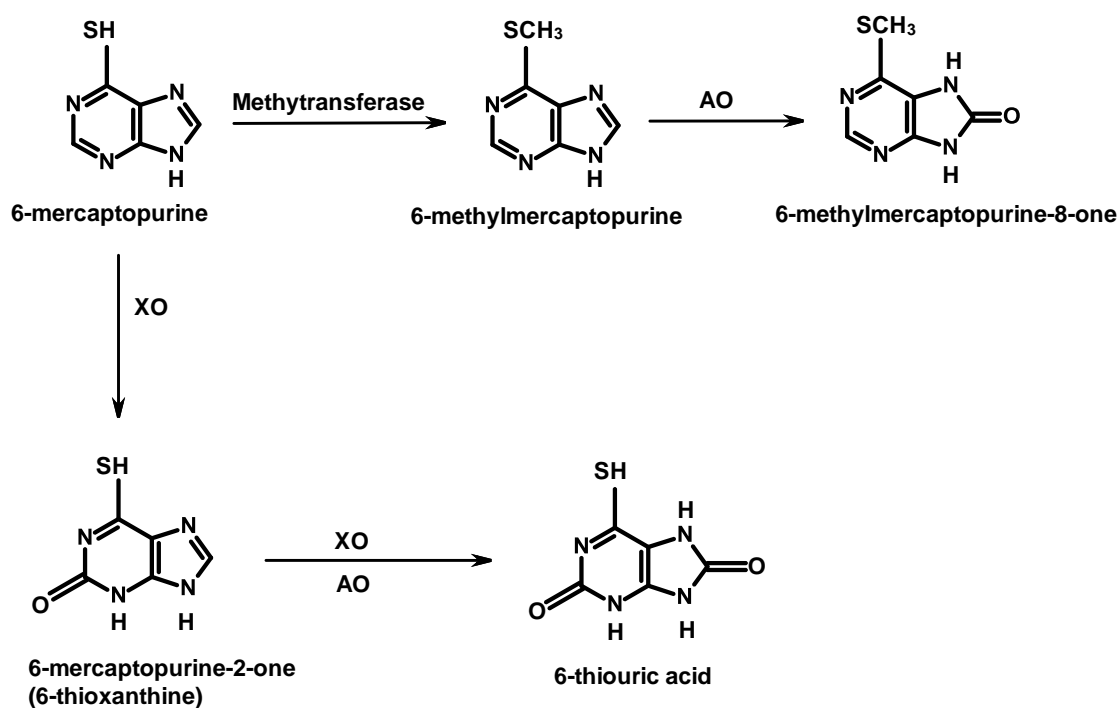


Figure 8: The oxidation of 6-mercaptapurine, a common substrate for aldehyde oxidase and xanthine oxidoreductase to different oxidative products. Based on (Beedham, 2001; Kitamura *et al.*, 2006; Rashidi, 1996; Rashidi *et al.*, 2007; Zimm *et al.*, 1984).

In addition to the reactions outlined above AO and XOR can catalyse the oxidation of hundreds of other compounds (for reviews see Beedham, 2001, Kitamura *et al.*, 2006 and Garattini & Terao, 2012). As it is out with the scope of this thesis to list all these only compounds of that were chosen as targets for examination in this research study are listed in table 1.

Table 1: Examples of mammalian molybdo-flavo enzymes substrates.

Substrates	AO	XOR	References
1-methylxanthine (caffeine metabolite)	-	+++	(Krenitsky <i>et al.</i> , 1972; Reinke <i>et al.</i> , 1987)
3- methylxanthine (caffeine metabolite)	-	+	(Krenitsky <i>et al.</i> , 1972)
6-Mercaptopurine (anti-cancer drug)	+	++	(Beedham, 2001; Garattini & Terao, 2011, 2012; Kitamura <i>et al.</i> , 2006; Krenitsky <i>et al.</i> , 1972; Rashidi, 1996; Rashidi <i>et al.</i> , 2007; Zimm <i>et al.</i> , 1984)
7- methylxanthine (caffeine metabolite)	-	+	(Krenitsky <i>et al.</i> , 1972)
Allopurinol (hyperuricemia drug)	+	+++	(Beedham, 2001; Garattini & Terao, 2011, 2012; Kitamura <i>et al.</i> , 2006; Krenitsky <i>et al.</i> , 1972; Krenitsky <i>et al.</i> , 1986; Moriwaki <i>et al.</i> , 1993; Reiter <i>et al.</i> , 1990; Yamamoto <i>et al.</i> , 1991)
Cinchonine (anti-malarial drug)	+++	-	(Beedham <i>et al.</i> , 1992; Fukiya <i>et al.</i> , 2010; Itoh <i>et al.</i> , 2006; Kitamura <i>et al.</i> , 2006)
Hypoxanthine (purine endogenous)	-	+++	(Cao <i>et al.</i> , 2010; Kitamura <i>et al.</i> , 2006; Krenitsky <i>et al.</i> , 1972)
N ¹ -Methylnicotinamide (endogenous compound)	+++	-	(Beedham, 2001; Kitamura <i>et al.</i> , 2008; Kitamura <i>et al.</i> , 2006; Krenitsky <i>et al.</i> , 1972; Shibata <i>et al.</i> , 1988; Sugihara <i>et al.</i> , 2006)
Methotrexate (anti-cancer drug)	+++	-	(Beedham, 1985, 2001; Chladek <i>et al.</i> , 1997; Johnson, 1967; Jordan <i>et al.</i> , 1999; Kitamura <i>et al.</i> , 1999; Kitamura <i>et al.</i> , 2006; Moriyasu <i>et al.</i> , 2006)
Paraxanthine (metabolite of caffeine)	-	-	(Lelo <i>et al.</i> , 1989)
Phenanthridine (environmental pollutant)	+++	-	(Beedham, 2001; Garattini & Terao, 2011, 2012; Rashidi, 1996; Rashidi <i>et al.</i> , 2011; Rashidi <i>et al.</i> , 1997; Stublely & Stell, 1980; Taylor <i>et al.</i> , 1984)
Phthalazine (representative of a class of N- heterocycles used and widely used in pharmacology industry)	+++	+	(Beedham <i>et al.</i> , 1990; Beedham <i>et al.</i> , 1995; Garattini & Terao, 2011, 2012; Kitamura <i>et al.</i> , 2006; Obach, 2004; Panoutsopoulos & Beedham, 2004; Stublely <i>et al.</i> , 1979)
Pyrazinamide (tuberculosis drug)	+	+++	(Kitamura <i>et al.</i> , 2006; Lacroix <i>et al.</i> , 1989; Moriwaki <i>et al.</i> , 1993; Shibutani <i>et al.</i> , 1999; Yamamoto <i>et al.</i> , 1991; Yamamoto <i>et al.</i> , 1987)
Pyridoxal (vitamin B6)	+++	-	(Garattini <i>et al.</i> , 2008; Garattini & Terao, 2011, 2012; Kitamura <i>et al.</i> , 2006; Krenitsky <i>et al.</i> , 1972; Schwartz & Kjeldgaard, 1951; Stanulovic & Chaykin, 1971)
Quinidine (anti-malarial drug)	+++	-	(Beedham, 1987; Beedham <i>et al.</i> , 1992; Itoh <i>et al.</i> , 2006; Kitamura <i>et al.</i> , 2006)
Retinaldehyde (vitamin A aldehyde)	+++	-	(Garattini <i>et al.</i> , 2008; Garattini & Terao, 2011, 2012; Huang <i>et al.</i> , 1999; Huang & Ichikawa, 1994; Kitamura <i>et al.</i> , 2006; Terao <i>et al.</i> , 2009)
Theobromine (primary methylxanthine in chocolate)	-	-	(Krenitsky <i>et al.</i> , 1972)
Theophylline (asthma drug)	-	+	(Krenitsky <i>et al.</i> , 1972)
Vanillin (present in food stuffs)	+++	-	(Beedham, 2001; Garattini & Terao, 2011, 2012; Obach, 2004; Panoutsopoulos, 2005; Panoutsopoulos & Beedham, 2004; Rashidi, 1996; Sahi <i>et al.</i> , 2008)
Xanthine (endogenous purine)	-	+++	(Krenitsky <i>et al.</i> , 1972; Krenitsky <i>et al.</i> , 1986; Yamaguchi <i>et al.</i> , 2007)

(+++ = high activity ++ = intermediate activity + = low activity - = no activity).

In addition to the oxidation of substrates both enzymes have reductase activity. In the presence of an adequate electron donor or strictly anaerobic conditions, the reduction rates are dependent on the concentration of electron donor (Beedham, 2001; Kitamura *et al.*, 2006; Obach, 2004). Beedham, (2001) reported that the reduction rates of XOR is much lower than AO in many of reactions. Examples of reduction reactions catalyzed by AO and XOR are summarised in tables 2 and 3.

Table 2: Reduction of various compounds by aldehyde oxidase.

AO substrates	AO generated metabolites	References
Fenthion sulfoxide	Fenthion	(Kitamura <i>et al.</i> , 1999)
Nitrated polycyclic hydrocarbons	Hydroxylamines	(Tatsumi <i>et al.</i> , 1986)
Nicotinamide-N-oxide, imipramine-N-oxide, cyclobenzaprine-N-oxide and s-(-)-nicotine-1-N-oxide	Their parent amines (nicotinamide and imipramine)	(Kitamura <i>et al.</i> , 2001; Kitamura & Tatsumi, 1984; Sugihara <i>et al.</i> , 1996)
Acetophenone oxime, salicylaldoxime and benzamidoxime	Corresponding oxo compounds and a ketimine	(Tatsumi & Ishigai, 1987)
Methyl red and orange	Cleavage of the azo linkage	(Kitamura & Tatsumi, 1983; Stoddart & Levine, 1992)
Aromatic and heterocyclic hydroxamic acids (e.g salicylhydroxamic acid)	Amides (salicylamide)	(Sugihara & Tatsumi, 1986)
Nitrosoamines (N-nitrosodiphenylamine)	Hydrazines (1,1-diphenylhydrazine)	(Tatsumi <i>et al.</i> , 1983)
Sulindac sulfoxide	Sulindac sulfide	(Duggan, 1981; Kitamura <i>et al.</i> , 2001; Pay <i>et al.</i> , 1980)
Zonisamide	2-sulfamoylacetylphenol	(Kitamura <i>et al.</i> , 2001)

Table 3: Xanthine oxidoreductase catalyzed reduction of various compounds.

XOR substrates	XOR generated metabolites	References
Nitrated polycyclichydrocarbons	Hydroxylamines	(Tatsumi <i>et al.</i> , 1986)
S-(-)-nicotine-1-N-oxide	Nicotine	(Sugihara <i>et al.</i> , 1996)
6-bromomethyl-(9H)-purine	6-methylpurine	(Porter, 1990)
Mitomycin C	2,7-diaminomitosene	(Gustafson & Pritsos, 1992; Pan <i>et al.</i> , 1984)
Doxorubicin	its semiquinone metabolites	(Barnabe <i>et al.</i> , 2002; Yee & Pritsos, 1997)

1.3. Molybdo-flavoenzyme structure

AO and XO are complex metalloflavoenzymes that contain one flavin adenine dinucleotide (FAD) two non-identical iron sulfur centers [2Fe-2S] and a molybdenum cofactor (Moco) as prosthetic groups (Borges *et al.*, 2002; Garattini *et al.*, 2008; Garattini *et al.*, 2003; Nishino, 1994; Palmer *et al.*, 1969). The active form of the molybdo-flavoenzymes enzymes are homodimers comprising of two identical subunits of approximately 150 kDa. This is schematically illustrated in figure 9, with each subunit divided into three distinct domains as follows: an N-terminus which has a 20 kDa domain with two [2Fe-2S] clusters, a 40 kDa flavin adenine dinucleotide (FAD) binding domain, and an 85 kDa C-terminal domain harbouring the Moco and the substrate-binding sites (Garattini *et al.*, 2003; Garattini & Terao, 2012; Hille, 2005; Hughes *et al.*, 1992; Kisker, 1997; Mendel & Bittner, 2006; Wollers, 2008).

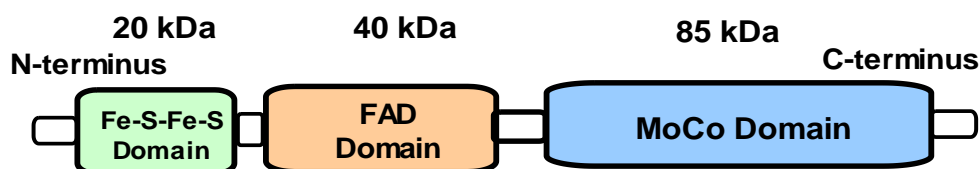


Figure 9: Schematic diagram of the domain structure of the molybdenum centre in enzymes of the XDH/AO enzyme family.

The N-terminus domain binds the two Fe_2S_2 clusters, the middle domain harbours the FAD binding site and the C-terminus domain is the location of the Mo-pterin cofactor (Mendel and Bittner, 2006).

A further insight into the domain structure of molybdo-flavoenzymes was provided by the successful crystallization of mammalian molybdo-flavoenzymes (Enroth *et al.*, 2000; Romao *et al.*, 1995; Truglio *et al.*, 2002). X-ray crystallography data determined that each subunit of the bovine XO and XDH enzymes may be separated into three sub-domains. The first sub-domain (residues 1-165) at the N-terminus contains both the iron-sulphur cofactors. It is associated with the FAD-binding domain (residues 226-531) by a long segment of amino acids (residues 166-225). Another linker segment (residues 532-861) then, connects this sub-domain to the molybdenum cofactor binding domain (residues 590-1,332), which is spatially located close to the interfaces of the iron sulphur and FAD binding domains (Figure 10) (Enroth *et al.*, 2000). Aldehyde oxidoreductase a related molybdenum-containing bacterial enzyme from *Desulfovibrio gigas* has also been crystallised and its structure reported at 2.25Å resolution (Rebelo *et al.*, 2000; Romao *et al.*, 1995). These crystal structures have unlocked a wealth of information revealing previously unknown structural details about these enzymes.

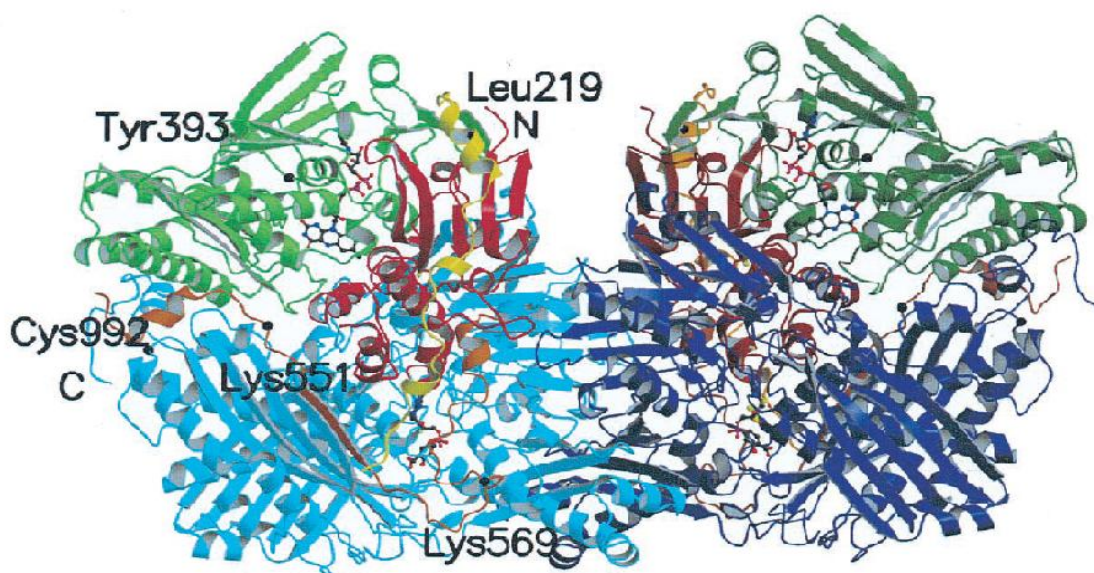


Figure 10: Crystal structures of bovine milk XOR dimer with the three domains represented in different colours. Iron sulfur-center domain (residues 3–165; red), FAD domain (residues 226–531; green) and Mo-pterin cofactor domain (residues 590–1331; blue) (Enroth *et al.*, 2000). The active site occurs between the two Lys569 and Lys551 residues. Used with permission from Professor Takeshi Nishino.

1.4. Molybdenum cofactor and the catalytic mechanism of molybdo-flavoenzymes.

Molybdenum itself is biologically inactive unless bound to a tricyclic pterin compound where it then forms molybdenum cofactor (Moco) (Figure 11). Moco is situated at the active site of all molybdenum enzymes (Mendel & Bittner, 2006). The pterin structure coordinates to the molybdenum metal by an enedithiolate side chain (Hille, 2005). The molybdo-flavo enzymes, AO and XO, contain an active site that is believed to be in a five-coordinate complex with two enedithiolate ligands of the molybdopterin cofactor, one oxo group, one sulfide group, and one hydroxyl or water molecule. This coordination results in a square pyramidal geometric structure and is the site for substrate binding and enzyme inhibition (Brondino *et al.*, 2005).

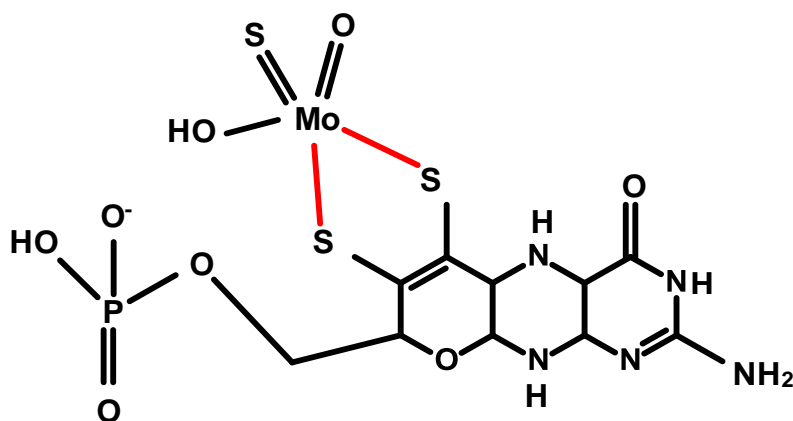


Figure 11: Molybdenum cofactor. The active site structure is attached to the pyranopterin compound by way of two enedithiolate side chains (red colour) to produce the molybdenum cofactor (Brondino *et al.*, 2005).

The function of the cofactor appears to be the transfer of electrons out of the molybdenum centre after the oxidation reaction has taken place. This results in the reduction of molybdenum from Mo (VI) to Mo (IV) (Hille, 2005). A proposed electron transfer sequence proceeds as shown below (Figure 12) (Hille & Nishino, 1995; Nishino & Okamoto, 2000), where Fe/S I and Fe/S II are two distinct iron-sulfur centers.

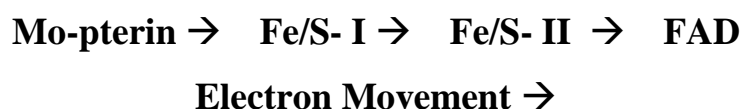


Figure 12: Proposed sequence of electron flow out of the molybdenum centre of molybdo-flavoenzymes. Based on Hille & Nishino, 1995; Nishino & Okamoto, 2000.

In general, mononuclear molybdo-flavoenzyme enzymes catalyze oxygen atom transfer reactions (Brondino *et al.*, 2005 ; Hille & Sprecher, 1987). In the case of molybdo-flavoenzymes, this is done through the hydroxylation of carbon centers of substrates containing N-heterocycle or aldehyde functional groups (Figure 13) where

the oxygen atom incorporated into the product ultimately comes from water and not from molecular oxygen (O₂), which is in contrast to a mono-oxygenase reaction (Hille, 2005).

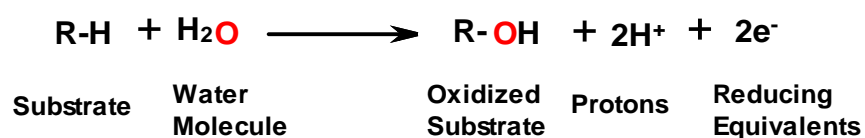


Figure 13: Oxygen atom transfer reaction by molybdo-flavoenzymes.

General oxidation reaction catalyzed by molybdo-flavoenzymes producing an oxidized substrate and two reducing equivalents (Beedham, 2001; Hille, 2005; Hille & Sprecher, 1987).

In the case of an aldehyde substrate the product is a carboxylic acid and xanthine the product is a uric acid (Figure 14).

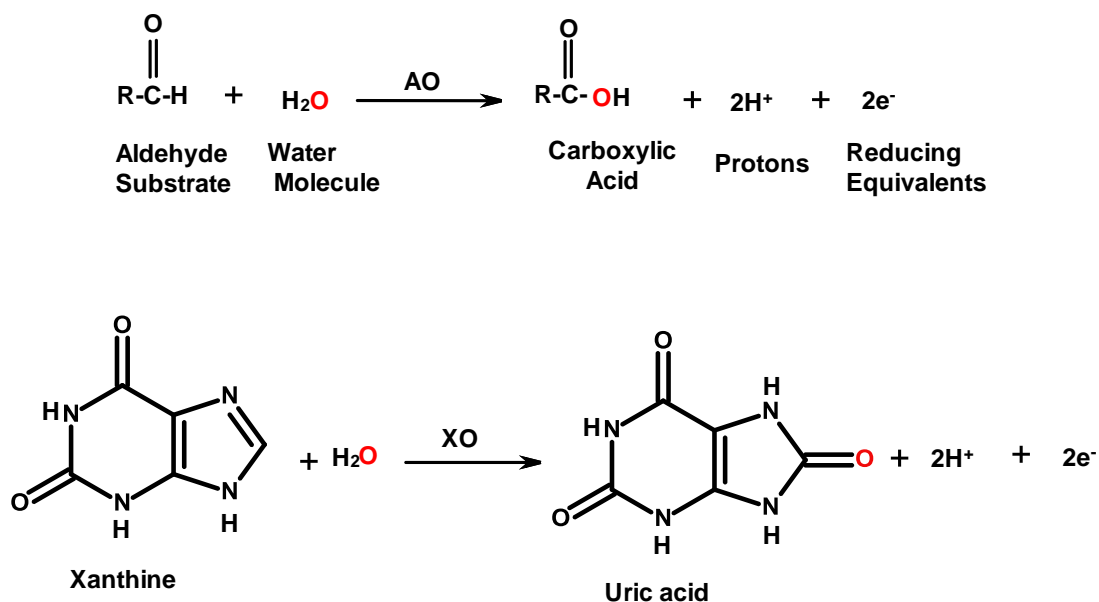


Figure 14: Oxygen atom transfer reaction molybdo-flavoenzyme involving an aldehyde and xanthine. Based on Hille & Nishino, 1995; Okamoto *et al.*, 2004.

The close relationship between AO and XOR implies a common mechanism of action for these two enzymes. Due to the increasing availability of crystal structures for some molybdo-flavoenzymes, the chemistry of the mechanism is now better understood (Choi *et al.*, 2004). There have been two proposed mechanisms for the xanthine oxidoreductase enzyme. The two mechanisms differ in the source of the labile oxygen atom. The alternate mechanisms illustrate either the oxygen from the oxo group (Mo=O) or the oxygen from the hydroxyl group (Mo-OH) as the catalytically labile (least stable) oxygen from the active site of the enzyme (Klaassen & Curtis, 2008; Xia *et al.*, 1999). The oxygen used as the labile one defines which oxygen atom is ultimately transferred to the substrate (Hille, 2002). Until about 1996, most researchers believed it was the Mo=O group that donated its oxygen to the substrate as an electrophile and the oxo ligand was revived from a water molecule (Fetzner, 2000; Hille, 2002). Subsequent experiments from other researchers have, however agreed with the evidence that supports the Mo-OH group as containing the labile oxygen (Bernhardt *et al.*, 2006; Choi *et al.*, 2004; Hille, 2005). The agreed mechanism using xanthine as the example substrate is shown in figure 15. This oxygen atom transfer reaction begins with an active site base abstracting the hydrogen from the Mo-OH group. The group then proceeds to initiate nucleophilic attack at the C-8 position of the xanthine substrate (Figure 15) (Choi *et al.*, 2004). The hydroxylation at C-8 position on xanthine then yields uric acid. At the same time, a hydride transfer takes place from the C-8 position on the xanthine substrate to the Mo^{VI}=S group. This then leads to a reduction of the molybdenum atom from (VI) to (IV) oxidation states. Loss of the proton from the sulphur and electron transfer produces transient formation of the Mo (V) intermediate. Water then enters the reaction, the oxidation product dissociates, and its position on the molybdenum centre is replaced by a water or

hydroxyl group. This then results in an increase in oxidation state of the molybdenum metal from Mo (V) to Mo (VI). In a similar manner AO can convert aldehydes to the corresponding carboxylic acid via a base-assisted nucleophilic attack of the Mo-OH on the substrate carbonyl with concomitant hydride transfer to the Mo=S (Figure 16).

An important amino acid residue that is involved in this process is glutamic acid. For the reaction mechanisms of XOR and AO, the O⁻ of Glu/1261 lies within a hydrogen bond length of the Mo atom (Cao *et al.*, 2011; Huber *et al.*, 1996; Metz & Thiel, 2009). This Glu residue has a role in catalysis as a Lewis-base, since it promotes the nucleophilicity attack of Mo-O⁻ on the carbon to be hydroxylated and concomitant hydride transfer to the Mo=S group to give an initial intermediate that that can be formulated as product (uric acid in the case of xanthine as substrate) (Cao *et al.*, 2011; Huber *et al.*, 1996).

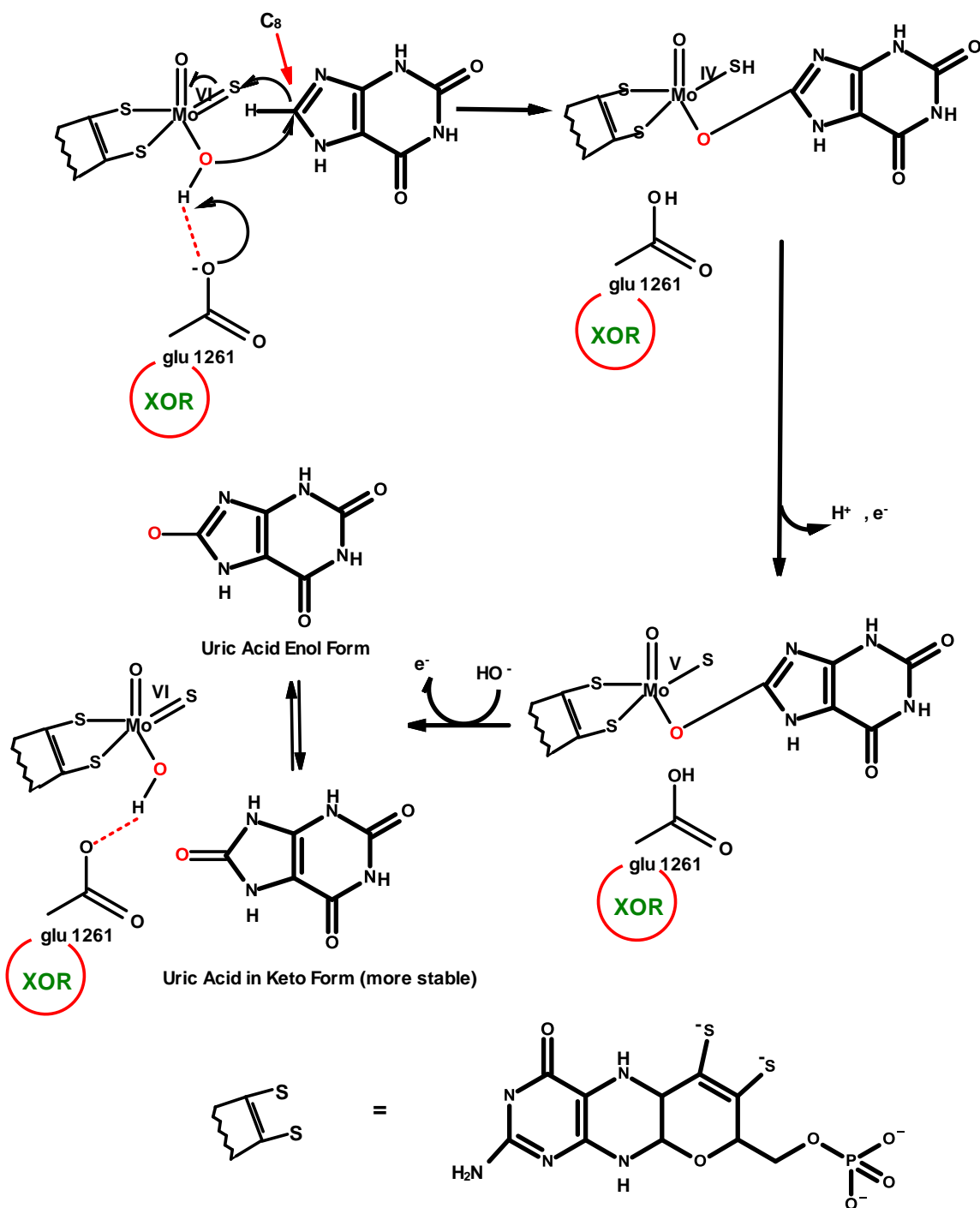


Figure 15: The reaction mechanism for XOR with xanthine as a substrate.

The active site base abstracts proton from the Mo-OH group which then undertakes nucleophilic attack on the C-8 position of the xanthine substrate with concomitant hydride transfer to Mo=S. Formation of paramagnetic species followed by displacement of product by hydroxide from solvent returns the active site to original state. The Glu residue important for the mechanism of catalysis is also shown (Choi *et al.*, 2004; Pauff *et al.*, 2009; Xia *et al.*, 1999).

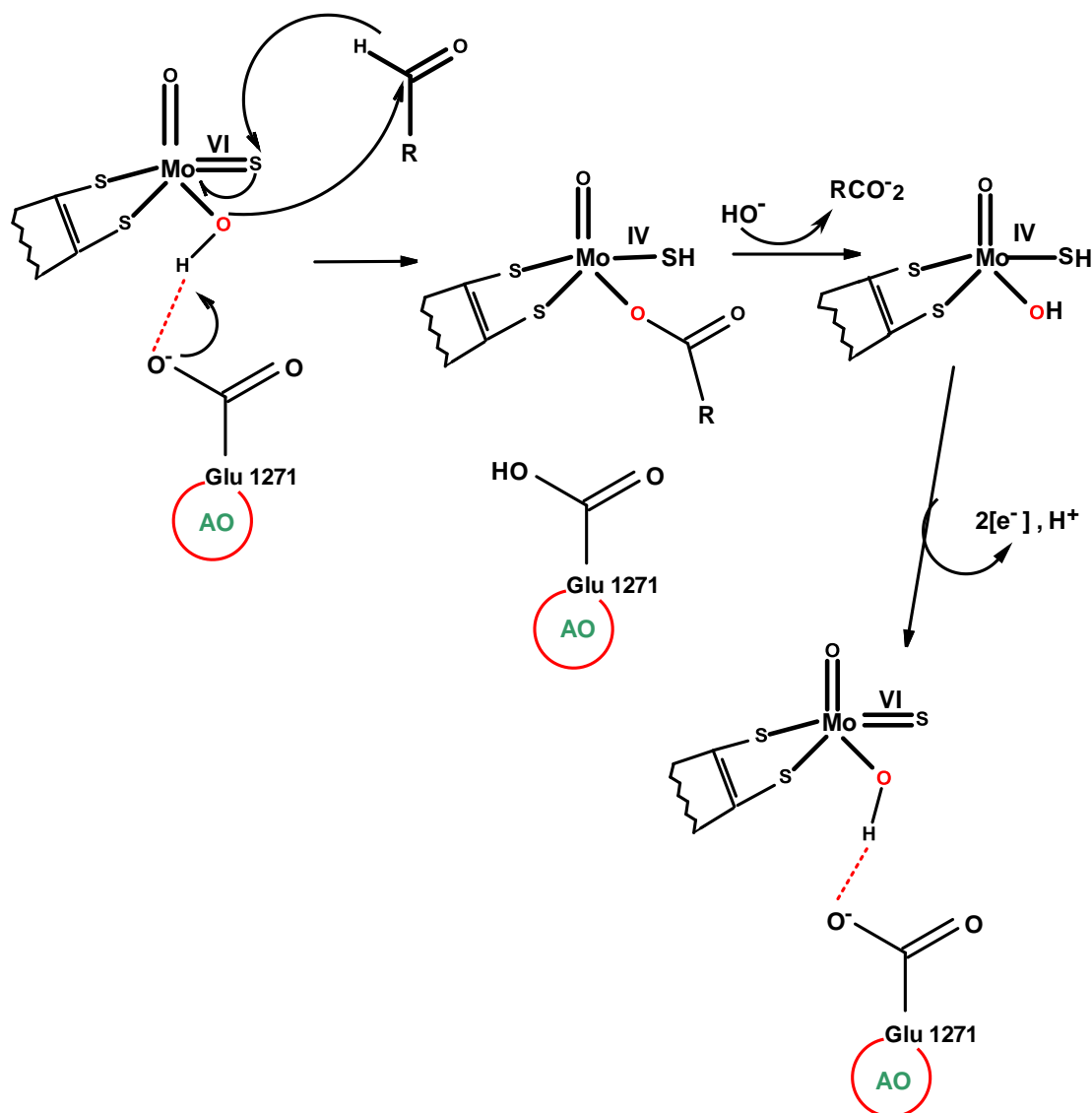


Figure 16: A general reaction mechanism of the AO enzyme with an aldehyde substrate.

A base-assisted nucleophilic attack of the Mo-OH on the substrate carbonyl with concomitant hydride transfer to the Mo=S which leads to the conversion of the aldehyde to its corresponding carboxylic acid. The Glu residue important for the mechanism of catalysis is also shown (Garattini *et al.*, 2008; Hille, 2005).

During substrate oxidation the molybdenum atom is initially reduced from Mo (VI), in oxidized enzyme to the Mo (IV) state to produce two electrons (Beedham, 1998; Bray, 1975; Hille, 2005; Hille & Nishino, 1995). *In vivo*, molecular oxygen serves as the electron acceptor for AO. XOR, on the other hand, exists primarily in the dehydrogenase form *in vivo* and its electron acceptor is NAD^+ (Beedham, 1998; Hille

& Nishino, 1995). The enzymes function by being alternately reduced by the substrate and then re-oxidised by their respective electron acceptors. *In vitro*, potassium ferricyanide and 2, 6-dichloroindophenol (DCIP) have proven to be effective artificial electron acceptors for these enzymes (Krenitsky *et al.*, 1972; Slef & Stadtman, 2000). Within the enzyme structure, flavin adenine dinucleotide (FAD) is the site where electrons are transferred to molecular oxygen where they produce either hydrogen peroxide (H_2O_2) by a two-electron reduction or a superoxide anion ($\text{O}_2^{\bullet-}$) by one-electron reduction in XOR and AO catalysis (Figure 17). With the XDH enzyme NAD^+ is reduced to NADH at the FAD site (Beedham, 1998, 2001; Hille, 2005; Hille & Nishino, 1995; Mendel & Bittner, 2006). These two pathways are illustrated below (Figure. 17).

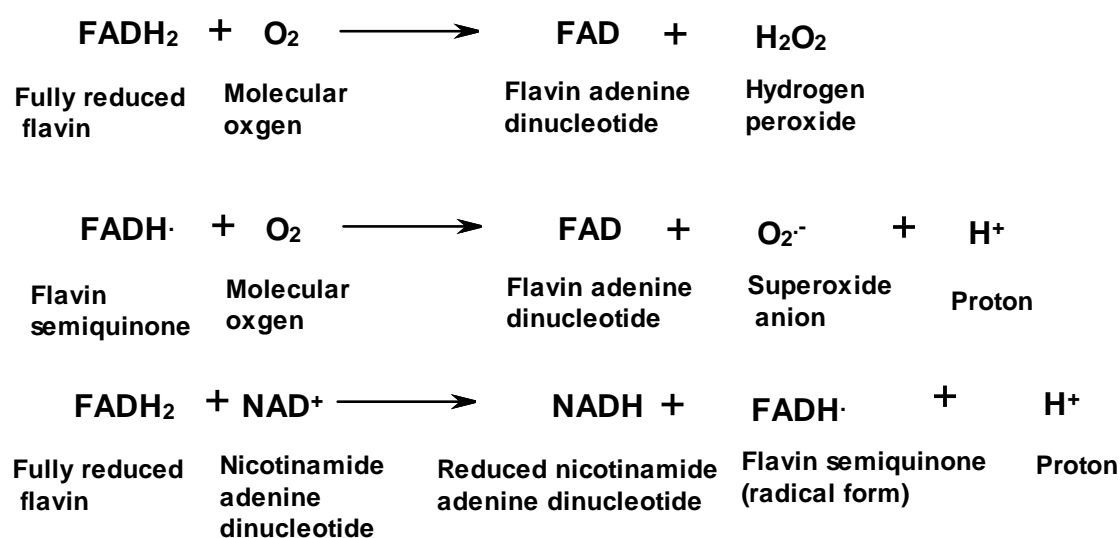


Figure 17: Reduction pathways occurring in molybdoflavoenzymes (Beedham, 1998; Hille, 1996).

The three different reduction pathways occur at the FAD site in the enzymes structure. The O_2 reactions occur with AO and XO. The NAD^+ reaction occurs with the XDH enzyme.

1.5. Species variation in molybdo-flavoenzyme activity.

AO and XOR are widely distributed among molluscs, crustaceans, insects, bacteria, fungi and all vertebrate classes (Beedham, 2001; Garattini *et al.*, 2008; Garattini *et al.*, 2003; Glatigny & Scazzocchio, 1995; Krenitsky *et al.*, 1972; Moura *et al.*, 1976; Pometto & Crawford, 1983; Romao *et al.*, 1995; Schrader *et al.*, 1999; Turner *et al.*, 1987; Woolfolk & Downard, 1977; Wurzinger & Hartenstein, 1974; Xu & Johnson, 1995; Yasuhara *et al.*, 2002). The activity of AO between animal species varies depending on the substrate considered. Sugihara *et al.* found the activity of AO in monkey is higher than human when using N¹-methylnicotinamide and benzaldehyde as substrates (Sugihara *et al.*, 1997). Klecker *et al.* (2006) found the activity of AO is highest in mouse towards zebularine substrate than monkey and human (Klecker *et al.*, 2006). Species differences have also found when using cinchonidine as a substrate where rabbits have higher AO activity than monkey (Fukiya *et al.*, 2010). In contrast, monkey AO has a higher reductase activity toward zonisamide as a substrate than rabbit (Kitamura *et al.*, 2001). Higher reductase activity was observed with rabbits AO towards naphthalene 1, 2-oxide and benzo [a] pyrene-4, 5-oxide as model substrates of epoxide compounds than the AO of rat (Hirao *et al.*, 1994). In contrast, Kitamura and Tatsumi (1984) found the AO reductase activity of rabbits, hogs, guinea pig, hamster, rat and mice had similar reduction activity towards nicotinamide N-oxide as a substrate (Kitamura & Tatsumi, 1984).

1.5.1. Vertebrate molybdo-flavoenzymes

The AOX and XOR genes of mammals are variable and species specific. Vertebrates are characterized by a number of active AO genes that ranges from 1 to 4 (Dickinson, 1971; Garattini *et al.*, 2008, 2009; Garattini *et al.*, 2003; Garattini & Terao, 2011, 2012; Lindsley & Zimm, 1992 ; Terao *et al.*, 2009; Terao *et al.*, 2006). Rodents (rat

and mice) have four of AO genes and one XOR gene (Garattini *et al.*, 2008; Garattini *et al.*, 2003; Garattini & Terao, 2011; Terao *et al.*, 2009). Of these two species and amongst all vertebrates mouse AOs are the best characterised. Table 4 summarizes the tissue distribution and substrate specificity of mouse AOs. In contrast to rodents other mammalian species have a reduced complement of functional MFE genes, bovine (*Bos taurus*) has three AO genes and one XOR, while dogs (*Canis familiaris*) have two AO and one XOR (Terao *et al.*, 2006). Humans have only one functional AO gene (AOX1) and one XOR gene (Garattini *et al.*, 2008; Garattini *et al.*, 2003; Garattini & Terao, 2012). The chicken (*Gallus gallus*) has two AO genes (AOX1 and AOH) and one XOR (Terao *et al.*, 2006). Interestingly, unlike humans and most other mammals, where AO activity is found in the liver, dogs and chickens do not express an AO enzyme in hepatic tissue. A summary of the current knowledge of MFEs in non-rodent species is given on table 5.

Table 4: Summary of the substrate specificity and tissue distribution of molybdo-flavoenzymes in *Mus musculus*.

The old nomenclature of AO enzyme for mouse (previous name) as used in the public databases (GeneBank) and publications up until 2008 and new nomenclature of mouse aldehyde oxidase adopted by Garattini *et al*, (2008) from 2008 onwards.

Old enzyme nomenclature of AO/XOR	New enzyme nomenclature of AO/XOR	Tissue distribution	Known substrates	References
AOX1	Aox1	Liver, lung, heart, kidney, testis, brain, eye, spinal cord and spleen	Butyraldehyde, nicotinamide, octanal, phenanthridine phthalazine, retinaldehyde, pyridoxal, vanillin, 2-hydroxypyrimidine	(Garattini <i>et al.</i> , 2008, 2009; Garattini <i>et al.</i> , 2003; Garattini & Terao, 2011, 2012; Kurosaki <i>et al.</i> , 1999; Kurosaki <i>et al.</i> , 2004; Moriwaki <i>et al.</i> , 1996; Vila <i>et al.</i> , 2004; Wright <i>et al.</i> , 1999)
AOH1	Aox3	Liver, lung and testis	Benzaldehyde, butyraldehyde, octanal, phthalazine, retinaldehyde, 2-hydroxypyrimidine	(Garattini <i>et al.</i> , 2008, 2009; Garattini <i>et al.</i> , 2003; Garattini & Terao, 2011, 2012; Kurosaki <i>et al.</i> , 1999; Kurosaki <i>et al.</i> , 2004; Moriwaki <i>et al.</i> , 1996; Terao <i>et al.</i> , 2001; Terao <i>et al.</i> , 2000; Tomita <i>et al.</i> , 1993; Vila <i>et al.</i> , 2004; Wright <i>et al.</i> , 1999)
AOH2	Aox4	Skin, stomach, Haderian gland, sebaceous gland, epidermis, eye, pancreas, brain and esophagus	Phenanthridine, retinaldehyde	(Garattini <i>et al.</i> , 2008, 2009; Garattini <i>et al.</i> , 2003; Garattini & Terao, 2011, 2012; Holmes, 1979; Kurosaki <i>et al.</i> , 2004; Sasai & De Robertis, 1997; Terao <i>et al.</i> , 2001; Terao <i>et al.</i> , 2000; Vila <i>et al.</i> , 2004)
AOH3	Aox11	Skin, Bowmans gland and olfactory mucosa	Benzaldehyde, octanal, phthalazine retinaldehyde, 2-hydroxypyrimidine	(Garattini <i>et al.</i> , 2008; Garattini & Terao, 2011, 2012; Kurosaki <i>et al.</i> , 2004; Terao <i>et al.</i> , 2009; Vila <i>et al.</i> , 2004)
XOR	Xdh	Liver, muscle, mammary glands, oesophagus, stomach, duodenum, jejunum, ileum, colon, rectum, lung, kidney, heart, small intestine and spleen	Hypoxanthine, xanthine	(Falciani <i>et al.</i> , 1994; Garattini <i>et al.</i> , 2008; Kurosaki <i>et al.</i> , 1995; Kurosaki <i>et al.</i> , 1996; McManaman <i>et al.</i> , 1999; McManaman <i>et al.</i> , 2002 ; Terao <i>et al.</i> , 1992; Terao <i>et al.</i> , 2006; Yoshimura & Oka, 1989)

Table 5: Summary of molybdo-flavoenzymes in non-rodent mammalian species.

Species	Tissue distribution of XOR	AO	Tissue distribution of AO	References
Human (<i>Homo sapiens</i>)	Liver, lung, mammary gland, kidney, spleen, stomach, skeletal muscle and milk	AOX1	Liver, lung and brain	(Beedham, 2001; Garattini <i>et al.</i> , 2008; Garattini <i>et al.</i> , 2003; Garattini & Terao, 2011, 2012; Krenitsky <i>et al.</i> , 1974; Minoshima <i>et al.</i> , 1995; Moriwaki <i>et al.</i> , 2001; H. Peretz <i>et al.</i> , 2007; Terao <i>et al.</i> , 1998)
Rhesus monkey (<i>Macaca mulatta</i>)	Liver, small intestine, brain and testis	AOX1, AOH2 and AOH3	Liver and nasal mucosa	(Fukiya <i>et al.</i> , 2010; Garattini <i>et al.</i> , 2008; Garattini & Terao, 2011, 2012; Hoshino <i>et al.</i> , 2007; Krenitsky <i>et al.</i> , 1974; Sugihara <i>et al.</i> , 1997; Tawa <i>et al.</i> , 2011)
Bovine (<i>Bos taurus</i>)	Liver, heart, mammary gland and milk	AOX1, AOH2 and AOH3	Liver, eye, kidney, testis, lung, spleen and ciliary body	(Beedham, 2001; Calzei <i>et al.</i> , 1995; Garattini <i>et al.</i> , 2008; Shimada <i>et al.</i> , 1989; Terao <i>et al.</i> , 2006)
Horse (<i>Equus caballus</i>)	Liver, skeletal muscle, lung and small intestine	AOX1, AOH1, AOH2 and AOH3	Liver	(Garattini <i>et al.</i> , 2008; Prichard <i>et al.</i> , 1991; Räsänen <i>et al.</i> , 1993; Seeley <i>et al.</i> , 1984)
Dog (<i>Canis lupus familiaris</i>)	Spleen, skin, nasal mucosa, lacrimal glands and oesophagus	AOH2 and AOH3	Nasal mucosa, lacrimal glands, testis, skin and oesophagus	(Garattini <i>et al.</i> , 2008; Krenitsky <i>et al.</i> , 1974; Terao <i>et al.</i> , 2006; Vila <i>et al.</i> , 2004)
Baboon (<i>Papio ursinus</i>)	Liver and intestine	AOX1	Liver, heart, kidney and intestine extract	(Garattini <i>et al.</i> , 2008; Holmes & Vandenberg, 1986)
Chicken (<i>Gallus gallus</i>)	Liver, lung, brain, skin, muscle, spinal cord, thymus, trachea, spleen, pancreas, stomach, adrenal gland and testis	AOX1 and AOH	Kidney brain, skin, muscle, thymus, trachea, lung, spleen, pancreas, stomach, adrenal gland and testis	(Garattini <i>et al.</i> , 2008; Nishino & Nishino, 1989; Nishino <i>et al.</i> , 1989; Terao <i>et al.</i> , 2006)

1.5.2. Insect molybdo-flavoenzymes

In *Drosophila melanogaster* XDH is a general component of peroxisomes (Beard & Holtzman, 1987) and is encoded by the rosy (*ry* or *Xdh*) gene (Tiveron *et al.*, 1991). AO and pyridoxal oxidase (PO) enzymes in *D. melanogaster* are encoded by *aldox* and *lpo* structural loci (Keller & Glassman, 1964) and were identified in many adult *Drosophila* tissues (Cypher *et al.*, 1982) using heptaldehyde and 2, 4, 5-trimethoxybenzaldehyde as specific substrates for AO and PO, respectively (see table 6) (Cypher *et al.*, 1982).

Table 6: Distribution of aldehyde oxidase and pyridoxal oxidase in adult *Drosophila melanogaster* tissues. + present and - : absent. Based on Cypher *et al.*, 1982.

Tissue	AO present	PO present
Brain	+	-
Corpora allata	+	-
Alimentary canal	+	-
Anterior midgut (ventriculus)	+	-
Cardia, corp and oesophagus	+	+
Malpighian band	+	-
Posterior midgut	-	-
Pyloric region	+	+
Rectal valve and rectum	+	-
Salivary gland	-	-
Salivary duct	+	-
Circulatory system	+	-
Hemolymph	+	-
Lymph glands	+	-
Nephrocytes and abdominal	+	-
Fat body	+	-
Endocyte	+	-
oviduct	+	+
Musculature	-	-
Spermatheca	+	+
Uterus	+	+
Vas deferens	+	+

As well as in addition to studies in *Drosophila*, AO has been found in antennae of tobacco hawk moth (*Manduca sexta*), cabbage armyworm (*Mamestra brassicae*), polyphemus silkworm (*Antheraea polyphemus*) and domestic silkworm (*Bombyx mori*) (Maibeche-Coisne *et al.*, 2004; Merlin *et al.*, 2005; Rybczynski *et al.*, 1989; Rybczynski *et al.*, 1990). In *M. sexta*, *A. polyphemus* and *B. mori* this AO is involved in the chemo-reception of pheromonal stimuli in the antennae by its transformation of aldehyde pheromones such as bombykal (Rybczynski *et al.*, 1989; Rybczynski *et al.*, 1990). In addition to enzyme activities XOR and AO genes have been investigated in several insects. *Drosophila melanogaster* has one XDH and four AO genes (Adams *et al.*, 2000; Dickinson & Gaughan, 1981; Garattini *et al.*, 2003) and silkworm (*Bombyx mori*) has two XDH and six AO genes (Komoto *et al.*, 1999; Pelletier *et al.*, 2007). The current knowledge of the numbers of XOR and AO genes and MFE substrates for insects are summarised in table 7. At present the association of substrate specificity with individual insect AO genes is unknown.

Common and scientific name	XOR gene	XOR substrates	AOX gene	AO substrates*	References
Cabbage armyworm (<i>Mamestra brassicae</i>)	-	-	<i>MbrAOX</i>	-	(Garattini <i>et al.</i> , 2008; Garattini <i>et al.</i> , 2003; Merlin <i>et al.</i> , 2005)
European blowfly (<i>Calliphora vicina</i>)	<i>CvXOR</i>	Hypoxanthine	-	-	(Merlin <i>et al.</i> , 2005; Rocher-Chambonnet <i>et al.</i> , 1987)
Fruit fly (<i>Drosophila melanogaster</i>)	<i>DmXOR</i>	Xanthine, 2-amino-4- hydroxypteridine, pterin, aminohydroxypteridine and salicylaldehyde	<i>DmAOX1-4</i>	Acetaldehyde, benzaldehyde, anisaldehyde, <i>p</i> -chlorobenzaldehyde, cinnamaldehyde, citral, citronellal, dimethylaminobenzaldehyde, heptaldehyde, hexaldehyde, <i>p</i> -hydroxybenzaldehyde, <i>m</i> -hydroxybenzaldehyde, <i>o</i> -hydroxybenzaldehyde, salicylaldehyde and vanillin	(Adams <i>et al.</i> , 2002; Bentley & Williamson, 1982; Browder & Williamson, 1976; Chovnick <i>et al.</i> , 1990; Courtright, 1967; Cypher <i>et al.</i> , 1982; Doyle <i>et al.</i> , 1996; Garattini <i>et al.</i> , 2008; Garattini <i>et al.</i> , 2003; Keith <i>et al.</i> , 1987; Reaume <i>et al.</i> , 1991; Rushlow & Chovnick, 1984; Wahl <i>et al.</i> , 1982; Wurzinger & Hartenstein, 1974)
Honey bee (<i>Apis mellifera</i>)	-	-	<i>AmelXDH</i>	-	(Pelletier <i>et al.</i> , 2007)
Mediterranean fruit fly (<i>Ceratitidis capitata</i>)	<i>CecaXOR</i>	-	-	-	(Merlin <i>et al.</i> , 2005; Pelletier <i>et al.</i> , 2007)
Mosquito (<i>Culex quinquefasciatus</i>)	<i>CqXOR</i>	Hypoxanthine	<i>CqAOX</i>	Acetaldehyde, benzaldehyde and heptaldehyde	(Coleman <i>et al.</i> , 2002)
Mosquito (<i>Anopheles gambiae</i>)	<i>AgamXDH</i>	-	<i>AgamAOX</i>	-	(Merlin <i>et al.</i> , 2005; Pelletier <i>et al.</i> , 2007)
Mosquito (<i>Aedes aegypti</i>)	-	-	<i>AaegAOX</i>		(Merlin <i>et al.</i> , 2005)
Silkworm (<i>Bombyx mori</i>)	<i>BmXOR1</i> and <i>BmXOR2</i>	Hypoxanthine	<i>BmAOX1-6</i>	Benzaldehyde and bombykal	(Garattini <i>et al.</i> , 2003; Kamoto <i>et al.</i> , 2003; Komoto <i>et al.</i> , 1999; Pelletier <i>et al.</i> , 2007; Rybczynski <i>et al.</i> , 1990; Terao <i>et al.</i> , 2001; Yasukochi <i>et al.</i> , 1998)

Table 7: Summary of known AO and XOR genes and substrates in insects. * Substrates for individual enzymes not known

1.6. Molybdenum cofactor biosynthesis and molybdenum cofactor sulfuration.

As well as the structural genes that encode AO and XOR there are also several genes involved in the biosynthesis of a sulfurated molybdenum cofactor that are essential for the formation of functionally active molybdo-flavoenzyme holoenzymes. MoCo biosynthesis (Figure 18) requires six gene products in humans, plants, and fungi (Garattini *et al.*, 2003; Mendel & Bittner, 2006; Mendel & Hansch, 2002) and involves a conserved pathway (Garattini *et al.*, 2003; Mendel & Bittner, 2006; Mendel & Hansch, 2002; Schwarz *et al.*, 2009), which can be divided into 4 stages in humans: The reaction in stage 1 involves the conversion of GTP to the sulphur-free pterin precursor Z (Hille, 2005; Mendel & Bittner, 2006; Schwarz *et al.*, 2009) mediated by MOCS1A and MOCS1B proteins (Mendel & Bittner, 2006; Reiss *et al.*, 1999; Reiss & Johnson, 2003). The second stage of MoCo biosynthesis requires the integration of two sulfur atoms into the precursor Z for the formation of in MPT dithiolate and is initiated by the enzyme MPT synthase (Mendel & Bittner, 2006; Reiss & Johnson, 2003) which contains two subunits, MOCS2A and MOCS2B, encoded in humans by the gene *mocs2* (Garattini *et al.*, 2003; Mendel & Bittner, 2006; Reiss, 2000; Reiss & Johnson, 2003). MPT is then activated by a sulfotransferase protein which is encoded by the gene *mocs3* (Johnson *et al.*, 1989; Reiss & Johnson, 2003). In stage 3, the incorporation of Mo into MPT, which necessitates the uptake of molbydate (the form in which organisms accept Mo), takes place, thus forming MoCo. Gephyrin (protein encoded by the gene *GEPH*) then catalyses the insertion of the molybdenum atom into the molybdopterin to create an active molybdenum cofactor suitable for use in sulphite oxidase (Garattini *et al.*, 2003; Hille, 2005; Mendel & Bittner, 2006; Reiss, 2000). Finally a sulphur atom needs to be added to MoCo in order for AO and XOR to utilise

the cofactor (Mendel & Bittner, 2006). This is achieved by the enzyme molybdenum cofactor sulfurase (MCS) (Ichida *et al.*, 2001).

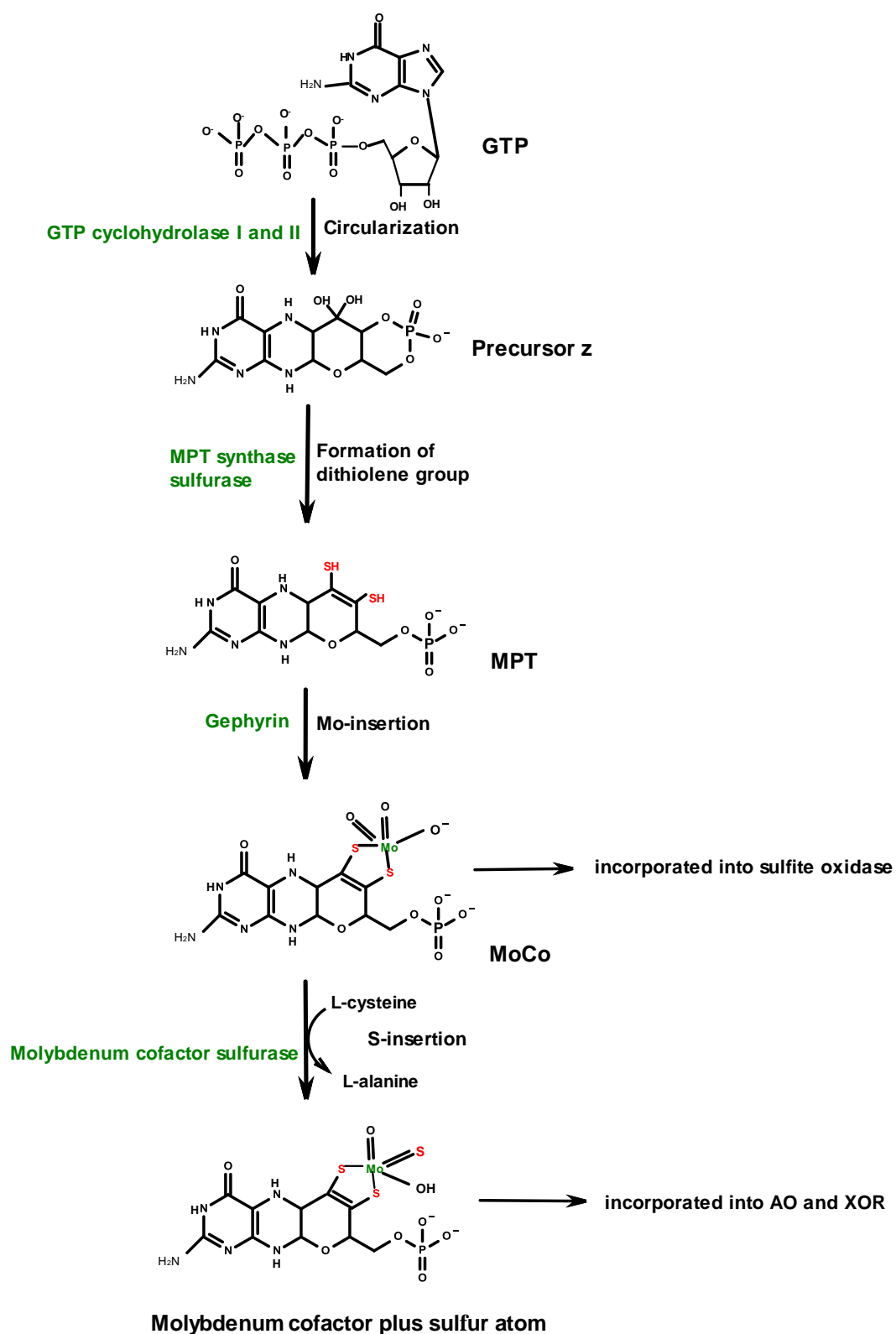


Figure 18: Summary of molybdenum cofactor synthesis based on Mendel & Bittner, 2006.

1.6.1. Identification of the molybdenum cofactor sulfurase gene and protein.

Molybdenum enzymes are divided into two subgroups. The first group contains enzymes with a dioxo-molybdenum centre (MoCo with two additional oxo-ligands and a protein-derived cysteinyl sulphur bound to the enzymes) like sulphite oxidase and nitrate reductase (NR) that carry two oxygen atoms at the molybdenum. The second group enzymes that contains AO and XOR have a monooxo-molybdenum centre and possess one oxygen atom, a terminal inorganic sulfur atom instead of the oxygen atom (Figure 19) (Hille, 1996; Mendel & Hansch, 2002) and one hydroxyl group ligated to the pterin-chelated molybdenum of the active enzyme (Wollers *et al.*, 2008). This unique sulfur moiety was discovered using maroon-like (*ma-l*) *D. melanogaster* mutants that are deficient in XDH and AO but retain SO activity. The *ma-l* mutant gave the first clue as to how sulphur incorporation occurred (Forrest *et al.*, 1956; Forrest *et al.*, 1961; Hille *et al.*, 2011) This was attributable to the requirement of a terminal sulphur ligand of the molybdenum cofactor by AO and XDH. In contrast, sulfite oxidase was unaffected in *ma-l* mutant because of the sulfite oxidase does not require a terminal sulphur ligand on the cofactor (Bogaart & Bernini, 1981; Hille *et al.*, 2011). The activities of AO, XOR and pyridoxal oxidase were reconstituting in the extracts of *ma-l* flies by resulphuration with sulfide/dithionite treatment (Wahl *et al.*, 1982). It was surmised that *ma-l* mutants had a lesion in post-translational sulfuration which was essential for the activity of AO, XDH and pyridoxal oxidase, but not of sulfite oxidase. Coughlan *et al.* found that the enzyme deficiency could be replicated by treating wild type enzyme extracts with cyanide. This suggested that sulphur is converted to thiocyanate in the presence of cyanide (Coughlan *et al.*, 1980) and was characterised to be an inorganic sulfide ion which is a terminal ligand of the molybdenum atom. As well as cyanide treatment spontaneous

loss of this sulfur also occurs under a variety of conditions generating a desulfo enzyme in which the terminal sulphide is replaced by a terminal oxo-ligand resulting in an inactive enzyme (Wahl & Rajagopalan, 1982). In addition to the earlier studies on *Drosophila* the activities of XDH and AO from crude extracts of *Arabidopsis thaliana* *Aba3* AO/XDH null-mutants was restored *in vitro* by anaerobic treatment with sulfite/dithionite, this indicated that these plants were also defective in the final sulfuration step of XDH and AO (Bittner *et al.*, 2001). As the sulfuration reaction was heritable this suggested evidence for the genetic control of the incorporation of the cyanolyzable sulfur of Mo hydroxylases. The protein responsible for the sulfur incorporation into the molybdenum cofactor of XDH and AO was named molybdenum cofactor sulfurase (Figure 19).

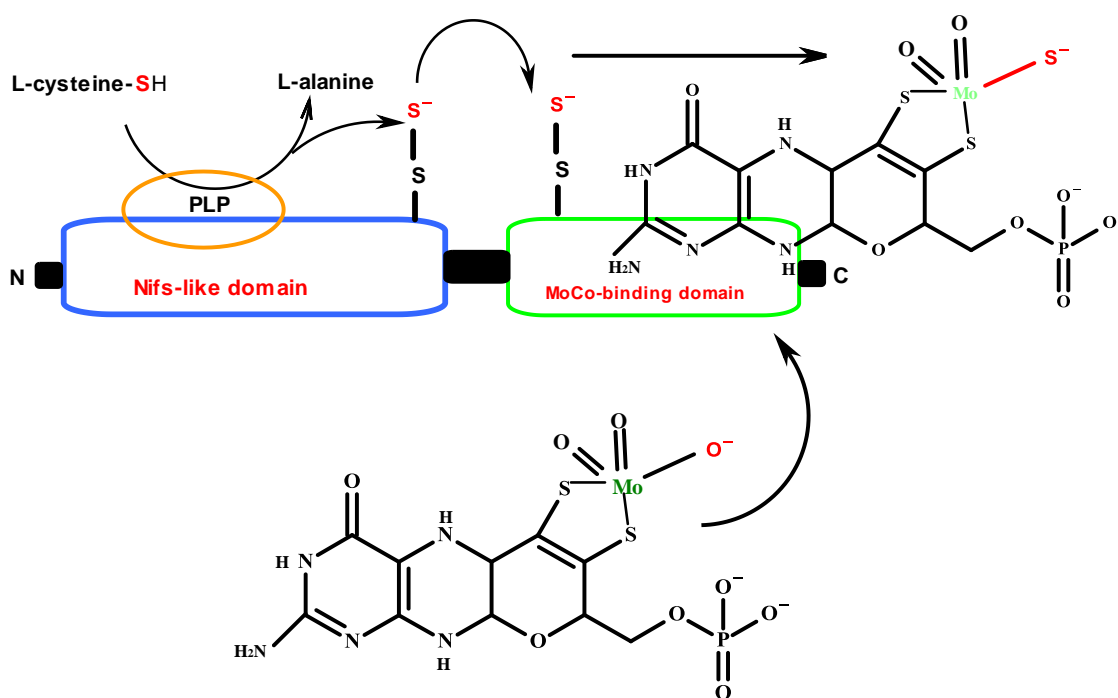


Figure 19: Domain structure and function of Moco sulfuryase *Aba3*. Based on Schwarz *et al.*, 2009, contains an N-terminal *NifS*-like domain that binds pyridoxal phosphate (PLP) via a conserved lysine residue and desulfurates L-cysteine to L-alanine, thereby generating a cysteine persulfide.

The persulfide sulphur generated by the NifS-like domain is transferred to the C-terminal domain for the conversion of bound desulfo-molybdenum cofactor into sulfurated molybdenum cofactor, which is required to activate XDH and AO enzymes.

It was not until 2000 that a gene and protein were identified that was responsible for this activity. In 2000 the *Drosophila melanogaster* and *Aspergillus nidulans* MCS cDNAs were cloned. The *Drosophila* gene was mapped to the X chromosome with a total length of 3.5kb with a coding region of 2.7kb spread over four exons (Amrani *et al.*, 2000). With the knowledge of the *Drosophila* gene homologous genes were cloned from other species. MoCo sulphurases have been cloned from bovine (*MCSU*) (Watanabe *et al.*, 2000), human (*HMCS*) (Ichida *et al.*, 2001), *A. nidulans* (*hxB*) (Amrani *et al.*, 1999) and *A. thaliana* (*Aba3*) (Xiong *et al.*, 2001). Of the MCS genes cloned the best studied is *Aba3* from the plant *Arabidopsis thaliana*. Comparison of

the NH₂-terminal domain of *Aba3* with other proteins indicated that it shares significant similarities to NifS-like cysteine desulphurases in a pyridoxal-5-phosphate (PLP)-dependent mechanism of trans-sulfuration (Bittner *et al.*, 2001; Hille, 2005; Hille *et al.*, 2011; Mendel & Bittner, 2006; Mendel & Hansch, 2002; Schwarz *et al.*, 2009) (Figure 20). The C-terminal domain does not exhibit striking similarities to any other protein apart from other molybdenum cofactor sulphurase in other species and a mitochondrial amidoxime-reducing enzyme (Havemeyer, 2006). Molecular analysis of three *Aba3* mutants in *Arabidopsis thaliana* identified mutations in both the N-terminal domain and C-terminal domain. These plants have strongly reduced XDH and AO activities but normal SO and nitrate reductase activities which are caused by a substitution of arginine 723 by a lysine within the C-terminal domain of the *Aba3* protein (Wollers *et al.*, 2008). The biochemical and pathological consequences of this genetic lesion was a lack of abscisic aldehyde to abscisic acid (Aba) conversion which related to simultaneous loss of AO and XDH activities, which resulted to total loss of seed dormancy, wilted phenotype and impaired stress response in the MCS deficient plants (Seo *et al.*, 2000). Further investigations demonstrated that the NH₂-terminal domain of *Aba3* transfers sulphur from L-cysteine to yield elemental sulphur, which is immediately bound as persulphide to a conserved protein cysteinyl residue within the domain (Wollers *et al.*, 2008). Recently Lehrke *et al.* (2012) confirmed these results by a structure of NifS-like domain of *Aba3* which indicated that Cys⁴²⁸ and Cys⁴³⁵ are conserved and bound as persulphide (Lehrke *et al.*, 2012). Co-incubation of *Aba3* C terminus carrying sulphurated molybdenum cofactor with partially active XDH and AO resulted in the stimulation of their enzymatic activities. This data suggested that the C-terminal domain of *Aba3* might act as a scaffold protein where pre-bound desulpho-molybdenum cofactor is converted into sulphurated cofactor prior to the

activation of XDH and AO (Wollers *et al.*, 2008). The domain structure and proposed mechanism of MCS is shown in figure 19.

1.7. Molybdo-flavoenzyme deficiencies.

Molybdo-flavoenzymes deficiencies with particular emphasis on hereditary xanthinuria, which is directly related to one of the research objectives described in this thesis.

1.7.1. Hereditary xanthinuria.

Xanthinuria is an inherited deficiency of XOR, which results in the inability to convert xanthine and hypoxanthine to uric acid (Dent & Philpot, 1954 ; Holmes & Wyngaarden, 1989). Hereditary xanthinuria can be subdivided into two types. Hereditary xanthinuria type I is characterised by a lack of XOR whereas the type II disease is characterised by a dual deficiency of AO and XOR. Hereditary xanthinuria type II is distinguished from hereditary xanthinuria type I by the oral administration of the AO substrates allopurinol, N¹-methylnicotinamide or pyrazinamide. Hereditary xanthinuria type I patients show a normal ability to oxidise these compounds to oxipurinol, 2- and 4-pyridonecarboxamides and 5-hydroxypyrazinamide respectively whereas patients with hereditary xanthinuria type II show a defective ability to oxidise these compounds indicating a dual deficiency of both AO and XOR because allopurinol and pyrazinamide are substrates for XOR and AO and N¹-methylnicotinamide substrate for AO (Levartovsky *et al.*, 2000; Reiter *et al.*, 1990; Yamamoto *et al.*, 1989; Yamamoto *et al.*, 1991). Hereditary xanthinuria type I is caused by a defect in the structural gene for XOR so only XOR is absent (Borucka *et al.*, 2010; Gok *et al.*, 2003; Simmonds *et al.*, 1995). 9 different mutations that cause hereditary xanthinuria type I have been identified that are summarised in table 8. With regards mutations that cause hereditary xanthinuria type II following the cloning of

the *Drosophila* MCS, the gene was identified in mammals by the study of a herd of cattle in Japan affected by xanthinuria type II. The bovine MCS gene has an open reading frame of 2547 nucleotides, which encodes a protein of 849 amino acids. This amino acid sequence was 40% identical to the *Drosophila* molybdenum cofactor sulfurase protein (Wahl *et al.*, 1982; Watanabe *et al.*, 2000). When compared to the sequence obtained from the affected offspring in the herd a three base pair deletion from nucleotides 769-771 resulting in the loss of tyrosine 257 was identified (Watanabe *et al.*, 2000). The following year the human MCS gene was cloned and a nonsense mutation that causes hereditary xanthinuria type II in two independent xanthinuric patients from the Kanto region of Japan identified (Table 8) (Ichida *et al.*, 2001). Later the first mutation in the C-terminal domain of HMCS was identified in a Bedouin-Arab child and a Jewish female (Peretz *et al.*, 2007). A list of known hereditary xanthinuria type II mutations is summarised in table 8. Mutations that lead to the genetic deficiency of XOR or dual AO/XOR deficiency in eukaryotes with their phenotypes are listed in tables 8 and 9.

Species	Disease/ Mutant	Gene/protein affected	Mutations	Symptoms
Humans (<i>Homo sapiens</i>)	Xanthinuria type I	Lack only XOR/XDH activity or mutation in gene encoding XOR	c.682C>T, resulting in p.Arg228X (Ichida <i>et al.</i> , 1998). c.2164A>T, resulting in p.Lys722Tyr in exon 20 (Gok <i>et al.</i> , 2003). A heterozygous deletion resulting in p.Pro214GlnfsX4 (Stiburkova <i>et al.</i> , 2012). p.Arg825X, p.R825X and p.R881X (Stiburkova <i>et al.</i> , 2012). c.445C>T, resulting in p.Arg149Cys (Sakamoto <i>et al.</i> , 2001). c.1658insC translation frameshift predicting a truncated protein of 569 amino acids with an altered C-terminal amino acid sequence as compared 1333 amino acids of the native protein (Levartovsky <i>et al.</i> , 2000). 514G>A resulting in p.Gly172Arg (Levartovsky <i>et al.</i> , 2000). 3326A>C resulting in p.Asp1109Thr (Levartovsky <i>et al.</i> , 2000). 3662A>G resulting in p.His1221Arg (Kudo <i>et al.</i> , 2008). c.2810C>T, resulting in p.Thr910Met (Arikyants <i>et al.</i> , 2007).	Xanthinuria and the accumulation of xanthine calculi in renal tubules that leads to renal dysfunction and hypouricemia results from decreased uric acid production.
Humans (<i>Homo sapiens</i>)	Xanthinuria type II	Lack of activity of both XOR and AO due to a mutation or mutations in the MCS gene	c.1255C>T, resulting in p.Arg419X (Ichida <i>et al.</i> , 2001). c.466G>C, resulting in p.Ala156Pro (Yamamoto <i>et al.</i> , 2003). c.1034insA resulting in p.Gln347fs*379, which led to an inactive HMCS protein because the truncated protein lacks the important Cys430 residue in the Nifs like domain, and the entire C-terminal domain (H. Peretz <i>et al.</i> , 2007). c.2326C>T, resulting in p.Arg776Cys. is essential for core structure of the C-terminal domain of the HMCS at this site may contribute clarifying the mechanism of MoCo sulfuration (H. Peretz <i>et al.</i> , 2007).	Xanthinuria and the accumulation of xanthine calculi in renal tubules that leads to renal dysfunction and hypouricemia results from decreased uric acid production. Appear a defective ability to oxidise allopurinol and pyrazinamide are substrates for XOR and AO and N ¹ - methylnicotinamide is substrate of AO indicating a dual deficiency of both AO and XOR
Bovine (<i>Bos taurus</i>)	Xanthinuria type II	Lack in both XOR and AO activity due to mutation in MCS gene	c.769-771delTAC resulting in p.Tyr257del (Watanabe <i>et al.</i> , 2000).	Xanthinuria and increased xanthine secretion in the urine related with lethal growth interruption at approximately 6 months of age.

Table 8: Genetic deficiency of mammalian molybdo-flavoenzymes.

Species	Disease/ Mutant	Gene/protein affected	Mutations	Symptoms
Fruit fly (<i>D.melanogaster</i>)	Model for xanthinuria type I (<i>rosy</i> mutants)	Lack XOR activity due to mutation in gene encoding XOR (<i>rosy</i> locus)	There are more than 200 <i>rosy</i> mutant strains are available, of which at least 23 have been sequenced. Four popular strains used are: 1) The <i>ry</i> ⁵⁰⁶ strain mutation is a deletion of approximately 2/3 of the 3'-end of the XDH gene (Hughes <i>et al.</i> , 1992; Reaume <i>et al.</i> , 1989). 2) The <i>ry</i> ⁶⁰⁶ strain p.Glu89Lys, this mutation is located in the iron-sulphur domain of the XDH gene (Gray <i>et al.</i> , 1991; Hughes <i>et al.</i> , 1992; Lee <i>et al.</i> , 1987). 3) The <i>ry</i> ⁵⁴⁴ strain p.Gly1011Glu, this mutation is located in the pterin molybdenum cofactor domain of the XDH gene (Gray <i>et al.</i> , 1991; Hughes <i>et al.</i> , 1992; Lee <i>et al.</i> , 1987). 4) The <i>ry</i> ⁴⁰⁶ strain p.Gly348Glu, this mutation is located in the flavin domain of the XDH gene (Gray <i>et al.</i> , 1991; Hughes <i>et al.</i> , 1992; Lee <i>et al.</i> , 1987).	Dull brown eye colour, which darkens with adult age due to a partial deficiency of the red pteridine pigments compared to relatively normal wild type.
Fruit fly (<i>D.melanogaster</i>)	Model for xanthinuria type II (maroon-like mutants)	Lack in both XOR and AO activity due to mutation in MCS gene	Unknown	Reddish brown eye colour from a reduction in the red (drosoplerin) pigments, loss in activity of XDH (Amrani <i>et al.</i> , 2000; Bentley & Williamson, 1982; Finnerty <i>et al.</i> , 1970; Forrest <i>et al.</i> , 1961).
Silkworm (<i>Bombyx mori</i>)	Model for xanthinuria type I	Mutation in <i>BmXDH</i> gene	The <i>og</i> ^z mutant has both an insertion and a deletion mutation. The deletion removes 386/822 amino acid residues (47% of the length) of the <i>og</i> protein, while the insertion inserts 47-bp, both at the C-terminal part, which caused premature stop codons and leads to the absence of XDH activity (Komoto, 2002; Komoto <i>et al.</i> , 2003).	Translucent mutant phenotype and accumulation of hypoxanthine and xanthine. High mortality during pupal period and low viability of moths. Causes sterility in females.
Silkworm (<i>Bombyx mori</i>)	Model for xanthinuria type II	Mutation in MCS gene	A 2.1 kb deletion containing the transcription initiation site, exons 1 and 2 the 5' end of exon 3 of the MCS gene was found in <i>og</i> ^z (Ozaki's-translucent) mutant, which caused a complete loss-of-function allele (Fujii <i>et al.</i> , 1998; Fujii <i>et al.</i> , 2010; Fujii <i>et al.</i> , 2009; Komoto, 2002).	Translucent mutant phenotype and accumulation of hypoxanthine and xanthine. High mortality during pupal period and low viability of moths. Causes sterility in females.
Thale cress (<i>Arabidopsis thaliana</i>)	Model for xanthinuria type II	Mutation in ABA3 MCS gene	Strong reduction in AO and XDH activities due to a substitution of p.Arg723Lys within the C-terminal domain of the ABA3 MCS protein (Heidenreich <i>et al.</i> , 2005; Sagi <i>et al.</i> , 2002; Wollers <i>et al.</i> , 2008).	Complete loss of AO and XDH activity. Loss of seed dormancy, loss stress tolerance and wilted phenotype
Fungi (<i>Aspergillus nidulans</i>)	Model for xanthinuria type II	Mutation in <i>hxB</i> gene	c.334delG of a methionine codon resulted in a stop codon 9 bp downstream (Amrani <i>et al.</i> , 2000; Anantharaman & Aravind, 2002; Yamamoto <i>et al.</i> , 1991).	Complete loss of AO and XDH activity. Unable to use hypoxanthine as a nitrogen source

Table 9: Genetic deficiency of non- mammalian molybdo-flavoenzymes.

1.8. Molybdo-flavoenzyme deficiencies in insects

AO and XOR enzyme deficiencies have been identified in two insect species, silkworm (*Bombyx mori*) and the fruit fly (*Drosophila melanogaster*) (Chovnick *et al.*, 1971; Chovnick *et al.*, 1990; Edwards *et al.*, 1977; Fujii *et al.*, 2009; Komoto, 2002; Komoto *et al.*, 2003). Although all the mutants are deficient in XDH activity, they are different from each other in molybdo-flavoenzyme activity. *Drosophila melanogaster* have been investigated for a number of years and first mutant that lacked both molybdo-flavoenzymes were discovered in 1956 (Forrest *et al.*, 1956; Hadorn & Schwinck, 1956). These mutants lack both XOR and AO activity and retain SO activity (Wahl & Rajagopalan, 1982). Maroon-like locus (*ma-l*) homozygotes are completely deficient in XDH and AO activities (Finnerty *et al.*, 1979; Kamdar *et al.*, 1997) and display a maroon eye colour compared to the bright red seen in wild type *Drosophila* (Amrani *et al.*, 2000; Finnerty *et al.*, 1979; Wahl & Rajagopalan, 1982). The two most widely used *ma-l* mutant strains (*mal-1* and *mal-f1*) were induced by X-ray and nitrogen mustard respectively (Bridgesc & Brehme, 1944; Schalet, 1960). Many studies reported that *mal-1* is able to complement *mal-f1*. Glassman and Pinkerton, (1960) as well as Ursprung, (1961) and Chovinck *et al.* (1968) have examined the complementation phenotype between *mal-f1/mal-1* they found low XDH and PO activity in the heterozygotes (Chovnick *et al.*, 1969; Glassman & Pinkerton, 1960; Ursprung, 1961). In insects the dual deficiency of XOR and AO has been extensively studied in silkworm. *Ozaki's translucent* (*og^z*) silkworm mutant strains with deletions, insertion and amino acid substitution in the MCS gene have been identified that lack AO activity along with XOR activity (Fujii *et al.*, 2009). The (*og^z*) mutation in the MCS gene leads to translucent larval skin because of the inability to synthesize uric acid, high mortality and sterility (see table 9) (Fujii *et al.*, 2009).

The *rosy* gene encodes the XDH apoprotein and *rosy* mutants show normal AO activity (Kidd *et al.*, 1999). The reason for these mutants being termed *rosy* relates to adult wild type flies have a bright red colour whereas *rosy* mutants have a brick red eye colour (see figure 20) (Cote *et al.*, 1986). Of the greater than 200 *rosy* strains of *D. melanogaster* currently available, at least 23 have been well characterised (Gray *et al.*, 1991).



Figure 20: Wild type eye colour on the left and *ry*⁵⁰⁶ mutant eye colour on the right.

Photograph taken by Dr DJ Clarke. Used with permission.

Maroon-like and *rosy* mutants both have the same dull red eye colour, are deficient in uric acid and have relatively high xanthine and hypoxanthine when compared with wild type strains. Maroon-like and *rosy* strains are unable to oxidise hypoxanthine levels to xanthine then to uric acid, or convert 2-amino-4-hydroxypteridine to isoxanthopterin, which are reactions catalyzed by the XDH. This is due to the absence of the XDH enzyme. In the *ry*⁵⁰⁶ strains the lack of the XDH enzyme is due to mutations in the XDH gene on chromosome 3 (see table 9) (Glassman & Mitchell, 1959). The genetic lesion is different in the maroon-like strain and is associated with the lack of a functional MCS gene on chromosome X (Courtright, 1967; Finnerty *et*

al., 1979). In addition, to the deficiency of XDH the *ma-l* mutants are also defective in AO (Courtright, 1967) and pyridoxal oxidase (Forrest *et al.*, 1961).

1.9. Effects of molybdo-flavoenzymes deficiencies on oxidative stress and xenobiotic toxicity in *D. melanogaster*.

1.9.1. Effect of molybdo-flavoenzymes deficiencies on oxidative stress and xenobiotic toxicity in *D. melanogaster*.

Uric acid has been shown to be an effective *in vitro* scavenger of singlet oxygen, peroxy radical and hydroxyl radical (Chow, 1988). As *ry* and *ma-l* strains cannot produce uric acid as they are deficient in XDH activity these strains provide a means for critical *in vivo* analysis of the role of uric acid in oxygen defence (Phillips & Hilliker, 1990). Biological models proposing an important antioxidant role of uric acid predict that urate-null mutants should be more susceptible to oxygen radicals. Hilliker *et al.* (1992) reported an investigation of the role of uric acid in oxygen defense in *ry*⁵⁰⁶ urate-null mutants. Hilliker *et al.* (1992) measured the toxic response of these mutants to oxygen stress imposed by exposure to radical generating redox cycling agents, ionizing radiation, and increased oxygen tension. The results clearly demonstrated the *in vivo* radical-scavenging role of urate and reveal a critical metabolic role of this classical molybdoenzyme-genetic system in *Drosophila*. Hilliker *et al.* reported that *ry*⁵⁰⁶ mutants are demonstrably impaired in their capacity to detoxify the active oxygen generated by such diverse agents as paraquat, hyperoxia, and ionizing radiation (Hilliker *et al.*, 1992). In addition, Humphreys *et al* found *ma-l* strain had paraquat hypersensitivity due to the lack of XDH activity, which leads to absence of uric acid (Humphreys *et al.*, 1993). In this role, uric acid can act either by directly scavenging active oxygen species, in which case it is oxidized into a variety of products including allantoin, oxaluric acid and parabanic acid (Kaur & Halliwell, 1990) or by binding radical-generating transition metals into poorly reactive

complexes (Davies *et al.*, 1986). Hamatake *et al.* also demonstrated that the urate-null strain was more sensitive to environmental cigarette smoke (ECS) toxicity than wild type strains (Oregon-R) as assayed by survival and fecundity. They hypothesised that oxidative damage seems to be involved in the toxicity of ECS as uric acid plays a role as an important antioxidant in *Drosophila* (Hamatake *et al.*, 2009).

In *D. melanogaster*, caffeine has been shown to cause premature aging and a reduction in lifespan especially in males (Kuhlmann *et al.*, 1968). It also induces somatic damage and chromosome breakage (Kuhlmann *et al.*, 1968). Caffeine also interferes with DNA metabolism (Nigsch *et al.*, 1977) DNA replication (Boyd & Presley, 1974), maternal systems (Mendelson & Sobels, 1974) and is mutagenic (Bateman, 1969). Graf & Wurgler (1986) found that 4% w/v caffeine media killed approximately 75% of exposed flies (Graf & Wurgler, 1986). Nikitin *et al.* 2008 showed that the lifespan of Canton-S *Drosophila melanogaster* males is reduced with varying caffeine concentration (1.5-13 mM) (Nikitin *et al.*, 2008). Zimmering *et al.* 1977 that found that caffeine is lethal to adult *D. melanogaster* (Zimmering *et al.*, 1977) and at lower concentration decreases longevity and fecundity in *Drosophila prosaltans* (Carrillo & Gibson, 2002; Itoyama *et al.*, 1998). Zimmering *et al.* (1977) found a high mortality rate (100%) in the *ry*⁵⁰⁶ flies after three days exposure to 1.25-2.5 mg/ml caffeine compared with wild type Canton-S in which the mortality rate was around 40%. This suggests that *Drosophila* XDH is involved in the chemoprotection against caffeine.

1.9.2. Effects of molybdo-flavoenzymes deficiencies on longevity of *D. melanogaster*.

It has been proposed that uric acid has important role as an antioxidant *in vivo* and singlet oxygen scavenger properties and it is important in determining species

longevity (Ames, 1983). A number of longevity investigations have been carried out with *Drosophila* strains that are XOR-null. In 1989 Shepherd *et al.* (1992) observed that the mutant ry^{506} strain had a reduced lifespan when compared to a wild type strain because the mean survival for male ry^{506} was approximately half of wild type (ry^+) (Shepherd *et al.*, 1989). A further study was carried out with XOR-null strain (ry^{506}), during this study male ry^{506} strains were observed to have a 20% reduced lifespan compared with the wild type strains (Oregon R). This study also demonstrated that ry^{506} flies had a reduced immune response to bacterial infection when compared to a wild type strain due to the important role XDH plays in the innate immune response (Kim *et al.*, 2001).

1.9.3. Effect of purine on molybdo-flavoenzymes deficiencies on *D. melanogaster*.

Geer and Laurie-Ahlberg tested survival rate of male wild type (Oregon R) and ry^{506} (null XDH) *D. melanogaster* in purine medium (Geer & Laurie-Ahlberg, 1984). Although the high concentration of purine was toxic to all strains, it was less toxic to the wild type than ry^{506} (Tiveron *et al.*, 1991). This demonstrated XDH role in chemoprotection against purine as a toxic substance.

1.10. Effect of molybdo-flavoenzymes deficiencies on temperature stress on *D. melanogaster*

The effects of temperature on the survival and longevity of *D. melanogaster* have also been investigated. Duncker serendipitously demonstrated the protection role of XDH in cold stress. Duncker *et al.* (1995) created transgenic *Drosophila* by transforming ry^{δ} flies that are deficient in XDH activity with an antifreeze protein (AFP) gene using a vector with the *xdh* marker gene to restore wild type eye colour. A control experiment was also performed in which the vector only without the AFP gene was used to transform the flies. When the two types of flies were tested for cold tolerance the

surprising finding was that flies that hosted the AFP gene did not have a greater tolerance than those with the vector only. Thus the AFP had no effect. However when the ry^8 were compared with transgenic flies with the vector it was found that they had a significantly increased temperature tolerance at 0 and -7°C implicating XDH as conferring this effect (Duncker et al., 1995).

1.11. Aims of the project.

The use of organisms that are genetically deficient in physiological functions has greatly aided our understanding of many biological functions. At present despite MFEs role as detoxification enzymes there has been no study to date that has used mutant organisms to authenticate this *in vivo*. Although bovine and human mutants that have hereditary xanthinuria exist, toxicological studies with these organisms are obviously problematic and raise ethical issues. Despite knockout mice that are XOR or AO deficient being created (Garattini *et al.*, 2008) they have not been used for such investigations and no dual mutants exist. The availability of *Drosophila melanogaster* that are models for both hereditary xanthinuria type I (XDH deficiency) and II (*ma-l* mutants with XDH and AO deficiency) provides an opportunity to scrutinize the role of these enzymes in detoxification and expand studies on their role in senescence and stress response. *Drosophila melanogaster* is ideal for such studies due to their well understood biology, short lifespan and low cost in carrying out experimentation.

The aims of this study are as follows:

- (a) Determine the molecular basis for dual deficiency of AO and XOR in *ma-l* mutants.
- (b) Determine the role of XOR and AO in the biotransformation of compounds *in vivo* and *in vitro* using MFE-deficient, transgenic and wild type *Drosophila melanogaster*.
- (c) Determine the role of MFEs in detoxification by examining the effects of exogenous and endogenous compounds in MFE-deficient, transgenic and wild type *Drosophila melanogaster*.
- (d) Determine the effect of MFEs deficiencies on lifespan and temperature stress in MFEs mutant, transgenic and wild type *Drosophila melanogaster*.

2.0. Materials and methods.

All chemicals and reagents were obtained from Fisher Scientific and Sigma / Aldrich Chemical Company Ltd, Poole, UK. Mobile phase reagents and solvents were obtained from various companies but were all for HPLC grade purity.

2.1. Animal studies.

2.1.1. *Drosophila melanogaster* strains used for research.

Drosophila melanogaster strains were obtained from the Bloomington *Drosophila* Stock Centre, Indiana University (USA). All these strains and mutations were those as described in (Lindsley & Zimm, 1992).

The following strains were obtained from Bloomington *Drosophila* Stock Centre:

Bloomington stock numbers are in parentheses

- Canton-S (1): Normal wild type *Drosophila melanogaster* (parental strain).
- ry^{506} (225): Strain deficient in XOR activity.
- *mal-1* (3973) and *mal-f1* (180): Strains deficient in MCS activity.
- Transgenic T1 and T2 strains: ry^{506} mutant strain transformed with a normal *Drosophila* XOR gene in Carnegie 20 vector.

2.1.2. *Drosophila melanogaster* media.

All fly strains stocks were housed at 25°C in controlled incubators with 12/12 h light/dark cycle and maintained on standard agar medium as follows:

Table 10: Chemicals used for preparing agar medium (Ashburner, 1989).

Chemicals	Amount for 250 ml of medium
Agar no. 2: (agar (15.0 g/L), peptone (vegetable) (5.0 g/L) and vegetable extract (3.0 g/L) from Sigma / Aldrich Chemical Company Ltd, Poole, UK	3.75g
Dried baker's yeast	21.5g
Tap water	250 ml
Sucrose	20.0 g
Antifungal chemicals	
Nipagen (Ethyl 4-hydroxybenzoate)	2.0 ml (2 g/20 ml in ethanol)
CBZ (Carbendazim solution)	7.5 ml (8 mg/ 100 ml in ethanol)

All the above ingredients with the exception of the anti-fungal compounds were weighed and dissolved in 250 ml of water and autoclaved. After autoclaving the medium, it was cooled to approximately 45⁰C and the antifungal chemicals were added and mixed. The medium was then poured into about 5 to 6 autoclaved bottles (height: 7 cm; diameter: 2.5 cm) (Figure 21) or 30 autoclaved vials (height: 12.5 cm; diameter: 5 cm) for lifespan and temperature stress studies. Care was taken to ensure no bubbles formed while pouring and that the media depth was approximately 3 cm in bottles and 1.5 cm depth in vials. Following aliquoting of media this was left to set and vials and bottles were kept at ambient temperature for 2 days covered with paper towels to ensure all moisture left on the side of the bottles was eliminated.

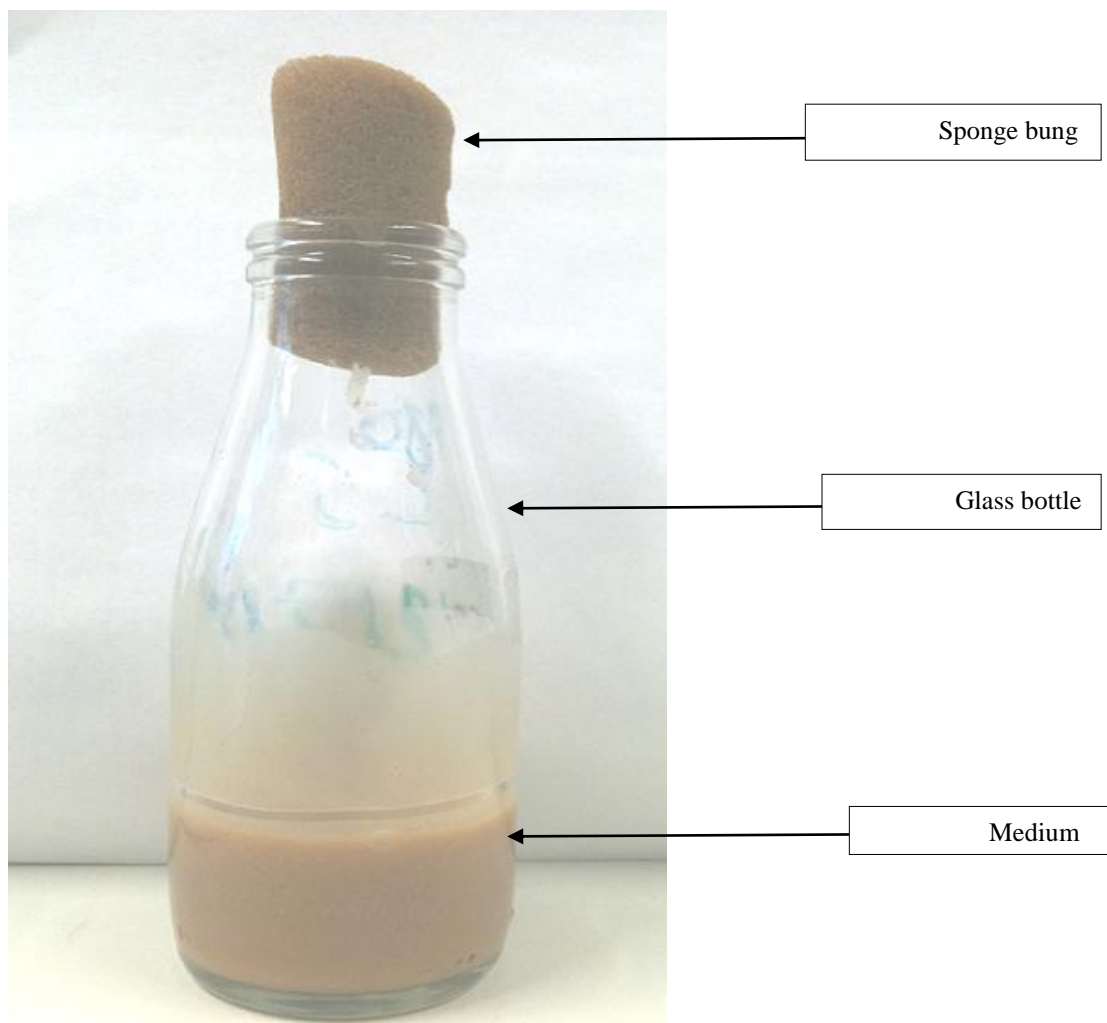


Figure 21: Bottle container for *D. melanogaster* media.

2.1.3. Procedure for transferring *Drosophila melanogaster* to new media bottles and vials.

A small amount of diethyl ether was poured in the base of the etherising container (Figure 22) and kept in it for few minutes so that it filled with ether vapour. After that, the glass bottle with *Drosophila melanogaster* was inverted over the funnel without the wooden cork so all *Drosophila* fell in to the funnel. To ensure the majority of flies were transferred the bottom of the bottle was slapped two times by hand. The plastic container and the funnel were then closed so that *Drosophila* did not fly out. Once all *Drosophila melanogaster* were etherised (usually after about 15-20 seconds), they were transferred into appropriate glass bottle or vial for further study. While etherising

the *Drosophila melanogaster* care must be taken so that they don't die. For maintenance of fly stocks new bottles of media were prepared weekly and all flies transferred.

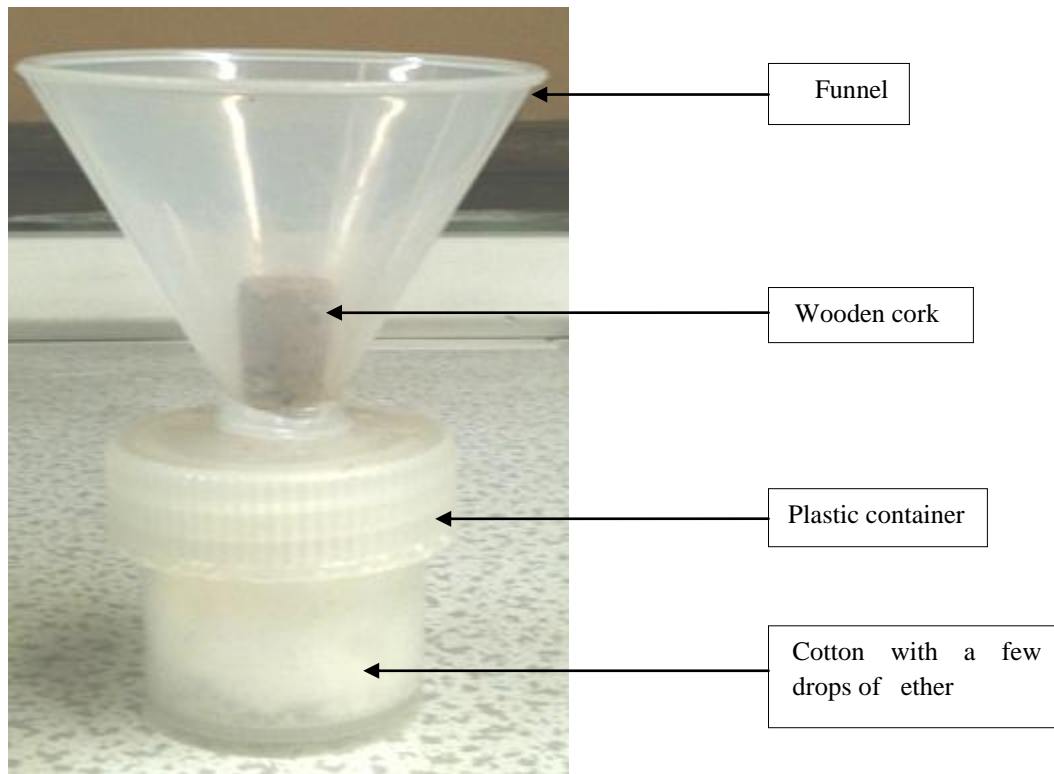


Figure 22: Container used for etherising flies.

2.2. *Drosophila melanogaster* lifespan tests.

Newly eclosed male wild type and mutant *Drosophila* strains were collected into glass vials containing approximately 5 ml of food medium as described in section 2.1.2. The vials were covered with sponge bungs and the flies were then left in medium vials overnight in a 25°C incubator to ensure they had recovered from the ether anaesthetic. Eclosing individuals were sequestered daily and male *Drosophila* were transferred to a fresh glass vial containing 5 ml of fly medium as described in section 2.1.3. Lifespan studies were carried out in a 12 hour light/dark cycle at 25°C to mimic the natural conditions of the *Drosophila*, with a total of 100 newly eclosed males (10 per vial)

tested for each longevity experiment. Bradley and Simmons (1997) studied ten different types of *Drosophila*; they found that lifespan increased when metabolic wastes did not exist in the media, so this factor affecting on the lifespan was corrected by frequent renewal of the media every 4 days (Bradley & Simmons, 1997). Transfer of flies into new vials with media was done by simply tapping the glass vial containing the flies on the bench so that all the flies dropped to the bottom of the vial, quickly removing the sponge bung and inverting the vial and gently tapping the flies into new vial. The new vial was then rapidly closed with a sponge bung.

2.3. Heat and cold shock experiments.

The same procedure was used as with the lifespan test except for the fact that 100 flies (3 days old) from each strain were placed in 5 vials with 20 flies per vial. These vials were sealed with a plastic leak-proof cap and immersed for 1, 2, 4 or 6 hours in a temperature ($-3 \pm 0.5^{\circ}\text{C}$, $0 \pm 0.5^{\circ}\text{C}$, $37 \pm 0.5^{\circ}\text{C}$ and $39 \pm 0.5^{\circ}\text{C}$) controlled circulating water bath to a depth such that vial's water seal cap plug was below water level (Golic & Lindquist, 1989). The controls from each strain were placed in the same sealed vials in an incubator at 25°C to make sure that there was enough oxygen after 6 hours. After each time the vials were removed and checked under the microscope for the number of flies paralysed (Gong & Golic, 2006). The flies were put into vials with media overnight to recover in a 25°C incubator. The next day the numbers of deaths, if any, were counted adult; flies that were able to fly, walk or stand were scored as alive (Overgaard & Sorensen, 2008).

2.4. *Drosophila melanogaster* toxicity tests.

All toxicity experiments were carried out at 25°C in a 12 hour light/dark cycle with 3 days old flies being used. The flies were collected and transferred to medium (see standard medium in section 2.1.2) containing different concentration of the AO and

XOR substrates used to evaluate chemoprotection by XOR and AO. All these substrates were made up in concentrations from 0 mM to 100 mM in distilled water or in appropriate solvents (Table 11). As the higher concentrations were close to the saturation point of some of the compounds the solutions were placed in a sonicating bath to increase dissolution of the compound. All the final concentrations of these xenobiotics were prepared by dilution with water before adding the dry ingredients for the normal culture medium (as standard medium in section 2.1.2). Care was taken to ensure uniform distribution of the chemicals by vigorously shaking in the culture medium. Vials with media were then left to dry for 2 days before use. For the experiments the vials were incubated at 25⁰C and the number of survivors recorded every 24 hours. Twenty flies were added to each vial and the survivors were transferred to new vials containing the same concentration of xenobiotics every four days. Control experiments were carried out using appropriate solvents as controls.

Table 11: A list of the xenobiotics used in toxicity experiments. Comments and appropriate solvents are indicated.

Xenobiotics (stock concentration)	Comment	AO and XOR substrate	Solvent
6-Mercaptopurine (100 mM)	Anti-cancer drug	AO/XOR	10 mM NaOH sonicating
Allopurinol (0.5M)	Hyperuricemia drug	XOR/AO	10 mM NaOH Sonicating
Caffeine (100 mM)	Drug and food industry	-	10 mM NaOH sonicating
Cinchonine (100 mM)	Anti-malarial drug	AO	dH ₂ O drops of 0.1M HCl
N ¹ -Methylnicotinamide (100 mM)	Endogenous vitamin metabolite	AO	dH ₂ O sonicating
Paraxanthine (0.5 M)	Metabolite of caffeine	XOR	10 mM NaOH sonicating
Phenanthridine (100 mM)	Environmental pollutant	AO	Absolute ethanol
Pyrazinamide (100 mM)	Tuberculosis drug	AO/XOR	10 mM NaOH sonicating
Theobromine (100 mM)	The primary methylxanthine in chocolate	XOR	10 mM NaOH sonicating
Theophylline (100 mM)	Asthma drug	XOR	10 mM NaOH sonicating
Vanillin (0.5 M)	Food industry	AO	dH ₂ O (heated to 70 ⁰ C)

2.4.1. Survival rate.

Survival rates were calculated for all strains by comparing results from control and xenobiotics supplemented medium for each strain.

Survival rate =

2.5. Analysis of the *in vivo* metabolism of molybdo-flavoenzyme substrates by *Drosophila melanogaster*.

Drosophila melanogaster (3 days old) were maintained on media containing various xenobiotics listed in table 11 and were removed from incubator and etherised. The whole flies were then immediately frozen at -80°C for HPLC analysis of metabolites. 20 flies weighing approximately 20 mg were placed in a 1.5 ml polypropylene Eppendorf tube and 180 µl of phosphate buffer saline (PBS) [0.2g of KCl 8g of NaCl 0.36g of Na₂HPO₄·2H₂O and 0.4g of KH₂PO₄ in 1000 ml of dH₂O, pH 7.4] was

added. This was homogenised with a plastic homogeniser, vortexed for 30 sec and centrifuged for 5 min at 10,000 xg. The supernatant was then removed and 20 µl was injected onto the HPLC. To confirm the individual peak retention time, 50 µl from each sample was spiked by mixing with 50 µl of 0.5 mM of working standard solution mixtures of individual compounds. Control assays were also performed on flies maintained on standard media without xenobiotics.

2.5.1. HPLC analysis of the xenobiotic substrates and metabolites.

Analysis of purine compounds, drugs and their metabolites were carried out by reverse HPLC. Using Beckman Coulter System Gold™ 127 Solvent HPLC Module (dual pump) and a programmable UV detector (module 166) or a programmable diode array detector (module 168) with injector equipped with a 20 µl sampling loop. The analytical columns used were either: a Kromasil (25 cm X 4.6 mm, 5µm; RP-C18), Hypersil ODS (25 cm X 4.6 mm, 5µm; RP-C18), LiChrosphere® (25 cm X 4 mm, 5µm; RP-C18) or Spherisorb® (5µm CNRP 4.6 X 15 cm; RP-C18). A guard column (5µm) with same stationary phase was used upstream of the analytical column. The columns were obtained from Phenomenex, UK and Captial HPLC limited, UK. The mobile phases were filtered through a 0.45µm filter before use. Isocratic and gradient system were used to develop the best conditions for the separation components and most highly sensitive method for the determination of the different compounds in biological samples. In order to achieve this, HPLC methodologies were developed to allow the necessary enzyme assays to be performed using only very small amounts of a sample that were available. The mobile phase reagents and solvents were all HPLC grade purity.

2.5.2. Analytical procedure.

2.5.2.1. Preparation of standards of calibration curves.

A 5 mM individual stock solution of AO and XOR substrates and products were prepared in appropriate solvent (see table 11). After preparing the stock solutions a working standard solution of 1 mM were prepared by dilution with water. Quantification was done using six standards prepared from stock solution with dH₂O ranging from 0.01 to 0.1 mM. These were injected onto the HPLC starting with the lowest concentration and were converted to (0.2 to 2 nmol/l); see figures 2-10 in appendix 1. All stock's solutions were stored at 4⁰C with the exception of vanillin and N¹-methylnicotinamide which were stored at -20⁰C.

2.5.2.2. Validation

2.5.2.2.1. Methods accuracy and precision.

Calibration standard injected on the same day were taken into calculation for intra-day accuracy and precision. This is a measure of the distribution of individual measurements around the mean. This parameter was assessed by repeated analysis of the same solution and expressed as the relative standard deviation (RSD) otherwise known as the coefficient of variation (CV). % RSD for retention time was calculated by dividing the standard deviation by the mean of retention time of three replicates in the same day (Ghassan *et al.*, 2010; Miller & Miller, 2005). The accuracy of the assay was calculated by comparison of the nominal analyte concentration to the actual concentration obtained from the linear regression line within the concentration range investigated (0.01mM to 0.1 mM) (Accuracy = nominal concentration / actual concentration x 100). By using slope, intercept and the correlation coefficient (RSQ or r²) for the linearity of the calibration curve was determined for each analyte.

2.5.2.3. Detection and Quantification limits.

For the linearity for the results, the lower limit of Quantification (LOQ) and limit of detection (LOD) were determined for each analyte. According to international conference on harmonisation (ICH) guidelines, the LOD is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. The LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy under the standard operational conditions of the method (ICH, 1994). The lower LOQ and LOD of samples was defined as the lowest added analyte concentration to the biological sample that can be measured with a low RSD and an accuracy of $100 \pm 20\%$ that can be discriminated significantly from the basal concentration of analyte in biological samples. The ICH has listed two options available to calculate both the LOD and the LOQ of an assay. One of these options are expressed as a concentration at a specified signal to noise ratio, usually 3:1 and 10:1 for the signal to noise ratio for LOD and LOQ respectively (ICH, 1994). The LOD and the lower of LOQ were calculated from the mean of the slope (S) and standard deviation (SD) of the intercept of three calibration curves using the liner regression linear method. The LOD and LOQ are calculated according to the following equations: The $LOD = 3.3 (SD/S)$ and the $LOQ = 10 (SD/S)$ (ICH, 1994; Miller & Miller, 2005).

2.5.3. HPLC assay of the purine based, N-heterocyclic and aldehydes substrates.

All endogenous purines and exogenous methylxanthine compounds (caffeine, theobromine, theophylline, paraxanthine and 6-mercaptopurine), purine analogs (allopurinol and oxipurinol), aldehydes (vanillin, retinal and pyridoxal) and N-heterocyclic compounds (phenanthridine, quinidine, cinchonine, pyrazinamide, methotrexate and N¹-methylnicotinamide) were purchased from Sigma Aldrich and reagents required for the preparation of the HPLC mobile phases were all of HPLC

grade purity. Initial stock solutions were prepared as 5mM solutions and were weighed into a flask and dissolved in appropriate solvents (see table 11). To ensure dissolution all stock solutions were sonicated for approximately 1 minute to dissolve any residue chromatograms in the solution. 1mM stocks were prepared by diluting the 5 mM stocks with distilled H₂O. Stock solutions were stored at 4C° in fridge. All mobile phases and HPLC methods for above compounds were summarized in tables 12 and 13.

Analyte	How analyte dissolved	Chromatograph column	Mobile phases	Gradient/ Isocratic	Flow rate ml/min	Wavelength	Reference
6-mercaptopurine and 6-thioxanthine	10 mM NaOH sonicating	Kromasil 5 μ m (25cm x 4.6mm, C18) with guard column 5 μ m	A: water H ₂ O with addition of 0.1% v/v of glacial acetic acid. Solvent B: acetonitrile	Gradient see figure 23	Flow rate was time programmed with the variation in the solvent which was as follows in figure 23	355 nm	Modified from (Begas <i>et al.</i> , 2007)
Allopurinol and oxipurinol	10 mM NaOH sonicating	Kromasil 5 μ m (25cm x 4.6mm, C18) with guard column 5 μ m	A: water H ₂ O with addition of 0.1% v/v of glacial acetic acid. Solvent B: acetonitrile.	Gradient see figure 24	Flow rate was time programmed with the variation in the solvent which was as follows in figure 24	280 nm	Modified from (Begas <i>et al.</i> , 2007)
Caffeine, theobromine, theophylline, paraxanthine, 1-methylxanthine, 3-methylxanthine and 7-methylxanthine	10 mM NaOH sonicating	Kromasil 5 μ m (25cm x 4.6 mm, C18) with guard column 5 μ m	A: water H ₂ O with addition of 0.1% v/v of glacial acetic acid. Solvent B: acetonitrile	Gradient see figure 23	Flow rate was time programmed with the variation in the solvent which was as follows in figure 23	280 nm	Modified from (Begas <i>et al.</i> , 2007)
N ¹ -methylnicotinamide	dH ₂ O sonicating	Water Spherisorb® 5 μ m (CNRP 4.6 x 150 mm; C18)	A: 0.1% v/v of formic acid B: acetonitrile	Gradient see figure 25	0.8	254 nm	(Szafarz <i>et al.</i> , 2010)
N ¹ -methylnicotinamide	dH ₂ O sonicating	Kromasil 5 μ m (25cm x 4.6mm, C18) with guard column 5 μ m	A: water H ₂ O with addition of 0.1% v/v of glacial acetic acid. Solvent B: acetonitrile	Gradient see figure 23	Flow rate was time programmed with the variation in the solvent which was as follows in figure 23	280 nm	(Begas <i>et al.</i> , 2007)
Phenanthridine and 6(5H)-phenanthridone	Absolute ethanol sonicating	Kromasil 5 μ m (25cm x 4.6mm, C18) with guard column 5 μ m	A: water H ₂ O B: acetonitrile.	Gradient see figure 26	1.5	254 nm	Modified from (LaVoie <i>et al.</i> , 1985).

Table 12: Summary of *in vivo* and *in vitro* HPLC analysis of purines, purines analog and N-heterocyclic substrates and products

Analyte	How analyte dissolved	Chromatograph column	Mobile phases	Gradient/ Isocratic	Flow rate ml/min	Wavelength	Reference
Cinchonine	dH ₂ O drops of 0.1M HCl	Hypersil ODS 5µm (25 cm x 4.6 mm, C18)	A: of 0.4% w/v CH ₃ COONH ₄ , pH 3.27 B: acetonitrile 35% v/v.	Isocratic	1.0	248 nm	(Beedham <i>et al.</i> , 1992)
Methotrexate	Absolute ethanol sonicating	Kromasil 5µm (25cm x 4.6mm, C18) with guard column 5µm	A: 0.1 M CH ₃ COONH ₄ , methanol (87:5 % v/v), pH 5.5 B: acetonitrile 8% v/v	Isocratic	1.5	305 nm	(Jordan <i>et al.</i> , 1999)
Pyrazinamide	10 mM NaOH sonicating	Kromasil 5µm (25cm x 4.6mm, C18) with guard column 5µm	A: 20 mM KH ₂ PO ₄ , pH 5.2 B: acetonitrile 5% v/v.	Isocratic	0.8	268 nm	(Kraemer <i>et al.</i> , 1998)
Pyrazinamide	10 mM NaOH sonicating	Kromasil 5µm (25cm x 4.6mm, C18) with guard column 5µm	A: water H ₂ O with addition of 0.1% v/v of glacial acetic acid. Solvent B: acetonitrile	Gradient see figure 23	Flow rate was time programmed with the variation in the solvent which was as follows see figure 23	268 nm	Modified from (Begas <i>et al.</i> , 2007)
Pyridoxal	dH ₂ O sonicating	Kromasil 5µm (25cm x 4.6mm, C18) with guard column 5µm	A: 60 mM Na ₂ HPO ₄ , pH 6.5 B: methanol 25% v/v.	Isocratic	0.8	315 nm	Modified from (Talwar <i>et al.</i> , 2003)
Retinal	Absolute ethanol sonicating	LiChrosphere® 5µm (25 cm x 4.0 mm, C18) with guard column 5µm	A: 30 mM CH ₃ COONH ₄ , pH 4.5 B: acetonitrile 70% v/v.	Isocratic	1.0	378 nm diode array detector	Modified from (Tsujita <i>et al.</i> , 1994)
Vanillin	dH ₂ O (heated to 70°C)	Kromasil 5µm (25cm x 4.6mm, C18) with guard column 5µm	A: 0.22 M KH ₂ PO ₄ , pH 2.9 B: acetonitrile 15% v/v.	Isocratic	1.5	285 nm	(Panoutsopoulos & Beedham, 2004)

Table 13: Summary of *in vivo* and *in vitro* HPLC analysis of N-heterocyclic and aldehyde substrates and products

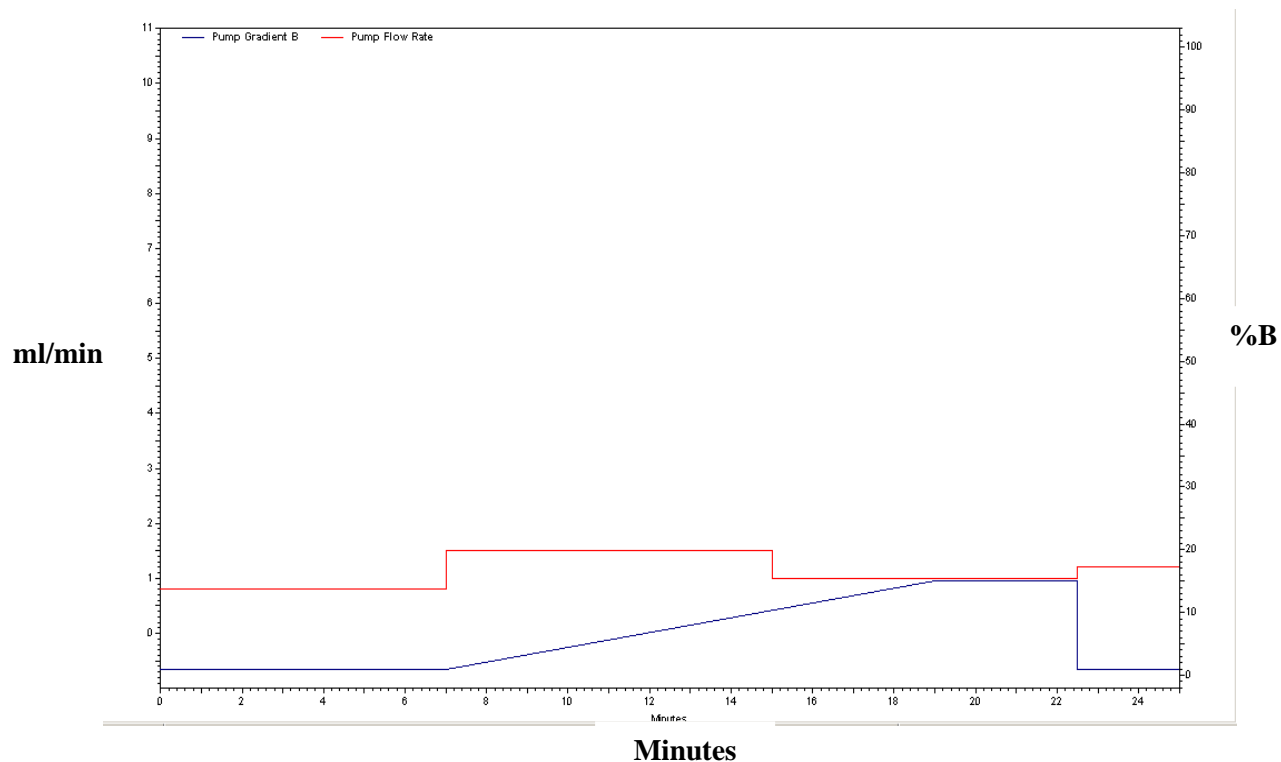


Figure 23: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for purines.

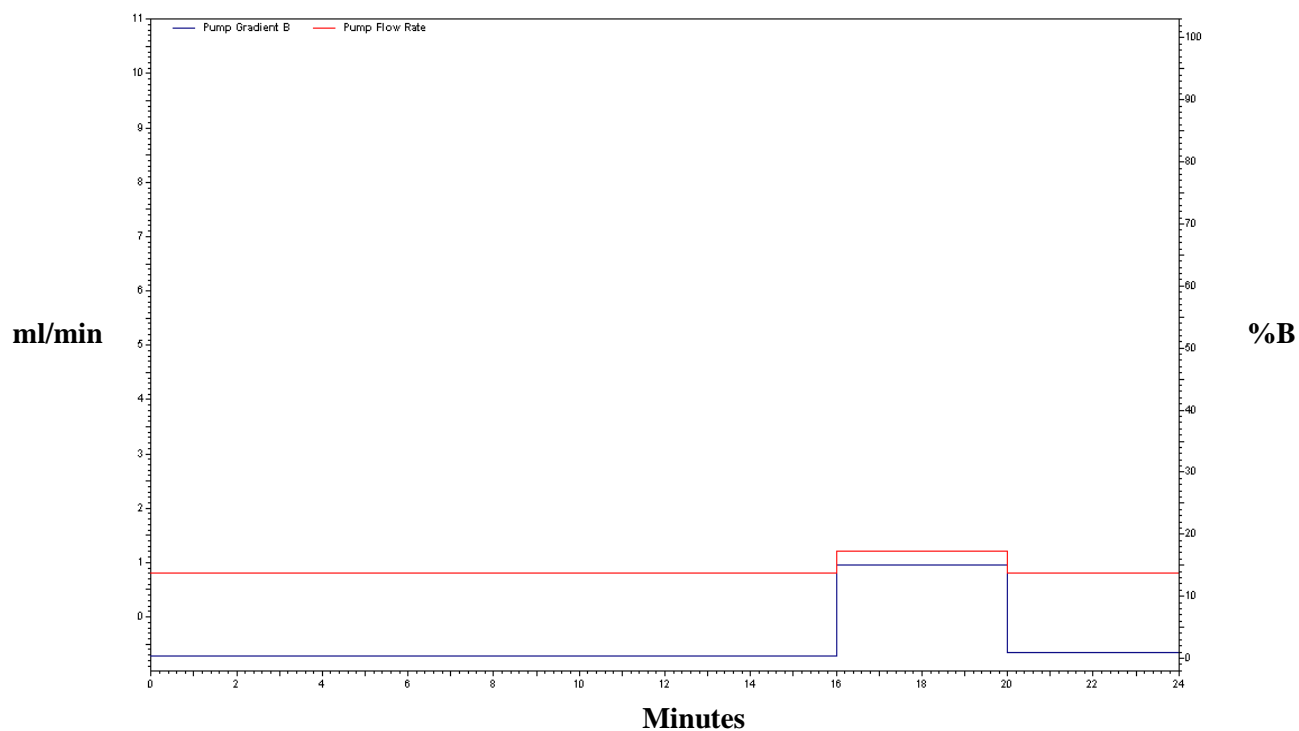


Figure 24: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for allopurinol and oxipurinol.

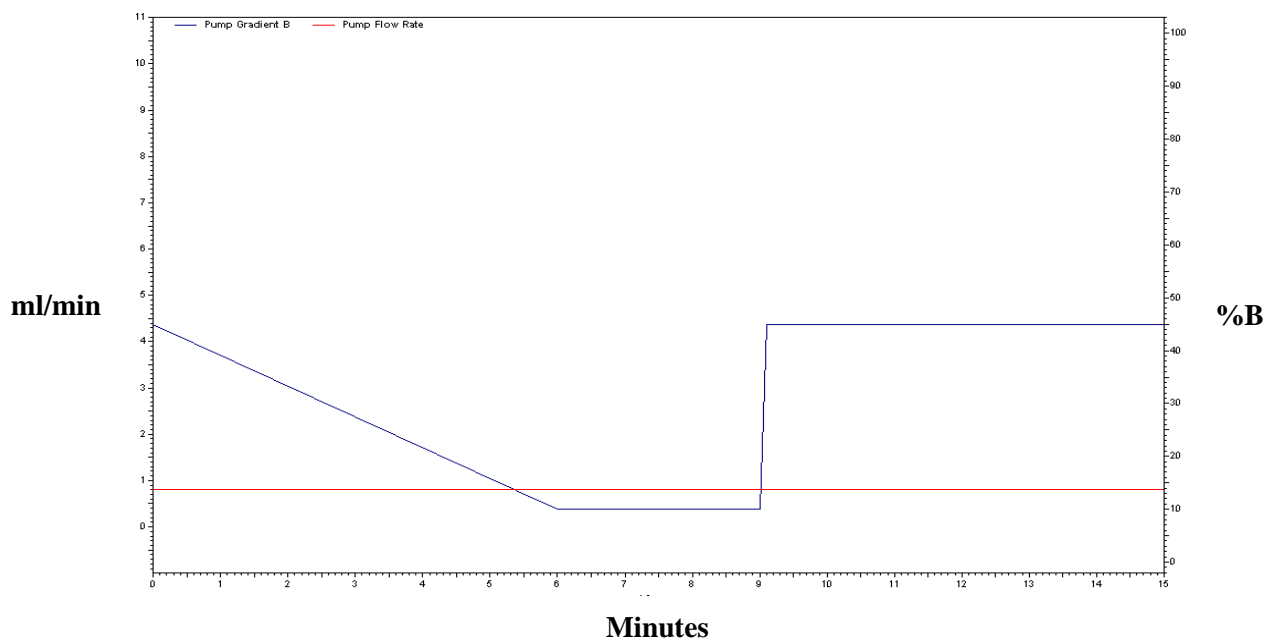


Figure 25: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for N¹-methylnicotinamide.

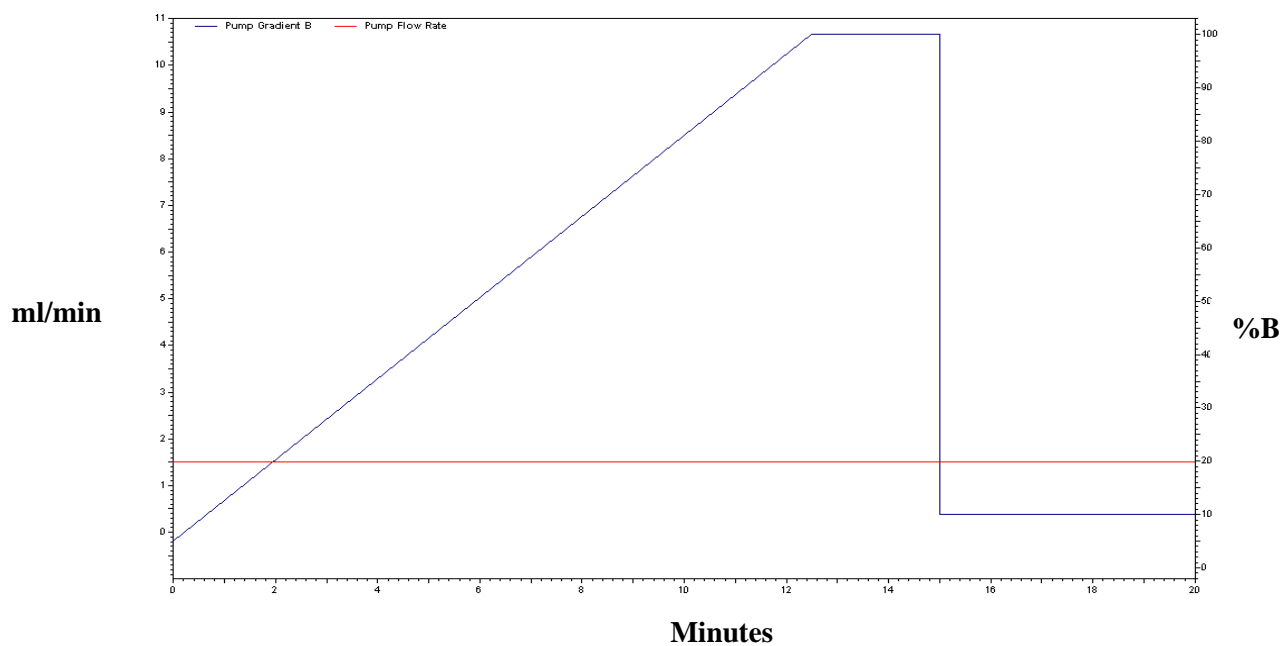


Figure 26: Diagram showing pump flow rate (red line) and pump gradient (blue line) change for phenanthridine and 6(5H)-phenanthridone.

2.5.4. *In vitro* analysis of *Drosophila melanogaster* molybdo-flavoenzymes activity.

2.5.4.1. Preparation of cytosol from *Drosophila*.

For the preparation of cytosol from *D. melanogaster* strains all steps were carried out at 0-4°C. Approximately 0.7 g of frozen flies from each strain were weighed out and homogenised in 2.8 ml of cold buffer (0.25 M sucrose, 10 mM Tris HCL pH 7.4) with a motor-driven tissue grinder fitted with a Teflon pestle to produce a 20% w/v homogenate. The homogenate was then centrifuged at 4°C for 15 minutes at 10,000 xg, to pellet out the exoskeletons and nuclear/mitochondrial fractions of the homogenate. The supernatant was then removed and centrifuged for a further 60 minutes at 4°C at 105,000 xg to obtain the cytosolic fraction. This fraction was then collected separated into 0.5 ml aliquots and stored at -80°C. The New Zealand white rabbit liver cytosol which was used to confirm the metabolite was prepared by Dr.D.J.Clarke.

2.5.4.2. Gel filtration of *Drosophila* cytosol.

Gel filtration was carried out in order to remove endogenous substrates such as purines and inhibitors, which may interfere with enzyme assays. A PD-10 gel filtration column was used (GE Healthcare Bio-Science, UK). Prior to use the column was allowed to drain before being equilibrated with 25 ml (5 column volumes) 50 mM Tris HCL pH 7.4. 1.5 ml of cytosol was then loaded onto the column and eluted with 2.5 ml of 50 mM Tris HCL pH 7.4, the protein containing fraction was then collected, pooled and aliquoted into 0.5 ml fractions in 1.5 ml polypropylene Eppendorf tubes to avoid repeated freeze/thawing and stored at -80°C.

2.5.4.3. Assessment of *in vitro* biotransformation of the purines, non-purines, N-heterocyclic and aldehydes substrates by *Drosophila* cytosol.

Individual AO and XOR substrates listed in table 14 were incubated with gel filtered *Drosophila* cytosol as indicated in table 14 at 37°C for various time periods. *In vitro* incubations were composed of 50 µl *D. melanogaster* cytosol of all strains, 30 µl phosphate buffer saline (PBS) and 10 µl 0.1 mM of all substrates listed in following table. 10 µl NAD⁺ (0.67 mM) was used as an electron acceptor for XDH enzyme assays. At the end of the incubation time all polypropylene Eppendorf tubes were transferred into ice to stop the reaction. For all analytes the effect of mixing the analytes with fly extract was assessed by spiking extract with an analyte. In all cases no difference in retention time was found. Spiking was also carried out to confirm peak identity as well as spectrum scanning using diode array detection when appropriate. In all *in vitro* assays no spontaneous oxidation of any substrate was observed when control incubations were carried out without cytosol.

Substrate	Stock substrate concentration	Volume of substrate added	Volume of cytosol	Volume of buffer	Volume of NAD ⁺ (6.7 mM)
1-methylxanthine	1mM	10 µl	50 µl cytosol	30 µl	10 µl
3-methylxanthine	1mM	10 µl	50 µl cytosol	30 µl	10 µl
6-mercaptopurine	1mM	10 µl	50 µl cytosol	40 µl	10 µl
7-methylxanthine	1mM	10 µl	50 µl cytosol	30 µl	10 µl
Allopurinol	1mM	10 µl	50 µl cytosol	40 µl	-
all- <i>trans</i> retinal	1mM	10 µl	50 µl cytosol	40 µl	-
Cinchonine	1mM	10 µl	50 µl cytosol	40 µl	-
Methotrexate	1mM	10 µl	50 µl cytosol	40 µl	-
N ¹ - methylnicotinamide	1mM	10 µl	50 µl cytosol	40 µl	-
Phenanthridine	1mM	10 µl	50 µl cytosol	40 µl	-
Pyrazinamide	1mM	10 µl	50 µl cytosol	40 µl	10 µl
Pyridoxal	1mM	10 µl	50 µl cytosol	40 µl	-
Vanillin	1mM	10 µl	50 µl cytosol	40 µl	-
Xanthine	1mM	10 µl	50 µl cytosol	30 µl	10 µl

Table 14: Summary of *in vitro* HPLC assays with molybdo-flavoenzyme substrates.

2.6. Protein determination.

The amount of protein in each sample was calculated using a modification of the method described by Smith *et al.* using bovine serum albumin (BSA) as standard (Smith *et al.*, 1985). The bicinchoninic acid (BCA) based assay is available as a kit from Sigma-Aldrich Co.

2.6.1. Bicinchoninic acid reagents.

Reagents supplied in the Sigma-Aldrich Co. kit are:

1. 1000 ml solution containing bicinchoninic acid, sodium carbonate (Na_2CO_3), sodium tartrate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$) and sodium bicarbonate (NaHCO_3) in 0.1N NaOH (final pH 11.25).
2. 25 ml solution containing 4% w/v copper (II) sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). For preparation of a set of protein standards a 1mg/ml stock solution of bovine serum albumin (BSA) was prepared and this was then used to make the dilutions to be used for the calibration curve as shown in table 15 and figure 1 in appendix 1.

Table 15: Dilution of BSA stock solution to give the required concentration to be used for the protein calibration standards.

Final protein concentration (mg/mL)	Volume BSA stock solution (μl)	Distilled water (μl)
0	0	100
0.2	20	80
0.4	40	60
0.6	60	40
0.8	80	20
1.0	100	0

2.6.2. Bicinchoninic acid methodology.

1. The required amount of protein determination reagent was prepared by adding 1 part $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 4% w/v solution to 50 parts bicinchoninic acid solution.

2. Bovine serum albumin standards or gel-filtered cytosol of *D. melanogaster* was diluted in duplicate at a 1:20 (20 µl cytosol + 380 µl PBS) dilution to get in middle of range of diluted standards.
3. 15 µl of freshly protein determination reagent was added to 300 µl of diluted cytosol and vortexed.
4. All tubes were incubated at 37°C for 30 minutes.
5. The absorbance at 562 nm was then measured on a microplate reader and distilled water was used to zero the instrument.

2.7. Spectrophotometric determination of molybdo-flavoenzymes activity.

Spectrophotometric molybdo-flavoenzyme assays were conducted using a microplate reader spectrophotometer (BioTek) at 37°C. All assays were carried out in triplicate in 100 µl reaction volumes. All cytosol samples were frozen and thawed only once, and the spectrophotometric data were collected at 5 second intervals for 3 to 5 minutes using Gen5™ software on a Windows XP PC connected to the microplate reader spectrophotometer (BioTek).

2.7.1. Spectrophotometric determination of dimethylaminocinnamaldehyde oxidase activity.

The activity of the AO enzyme was tested by monitoring the disappearance of oxidation of dimethylaminocinnamaldehyde (DMAC) at 398 nm (Kurth & Kubiciel, 1984). The reaction was carried out with 100 µl volume as summarized in table 16. The specific activity was calculated using the molar extinction coefficient for DMAC which is 30,500 M⁻¹cm⁻¹.

Table 16: Dimethylaminocinnamaldehyde oxidase assay.

Reagent	Volume	Final concentration
1 mM DMAC (dissolved in absolute ethanol)	10 μ l	0.1 mM
50 mM Tris HCl pH 7.4	50 μ l	25 mM
Gel-filtered <i>Drosophila</i> cytosol	40 μ l	

2.7.2. Spectrophotometric determination of phenanthridine oxidase activity.

The activity of the AO enzyme was tested by monitoring the oxidation of phenanthridine by an increase in absorbance at 322 nm (Johnson *et al.*, 1984). The reaction was carried out with 100 μ l as summarized in table 17. The specific activity was calculated using the molar extinction coefficient for phenanthridinone, which is 6,400 M⁻¹cm⁻¹.

Table 17: Phenanthridine oxidase assay.

Reagent	Volume	Final concentration
1 mM phenanthridine (dissolved in absolute ethanol)	10 μ l	0.1 mM
50 mM Tris HCl pH 7.4	50 μ l	25 mM
Gel-filtered <i>Drosophila</i> cytosol	40 μ l	

2.7.3. Spectrophotometric determination of vanillin oxidase activity.

The activity of the AO enzyme was tested by monitoring the disappear of oxidation of vanillin at 310 nm (Panoutsopoulos *et al.*, 2004). The reaction was carried out with 100 μ l volume as summarized in table 18. The specific activity was calculated using the molar extinction coefficient for vanillin, which is 8,854 M⁻¹cm⁻¹.

Table 18: Vanillin oxidase assay.

Reagent	Volume	Final concentration
1 mM vanillin (dissolved in hot water)	10 μ l	0.1 mM
50 mM Tris HCl pH 7.4	50 μ l	25 mM
Gel-filtered <i>Drosophila</i> cytosol	40 μ l	

2.7.4. Spectrophotometric determination of xanthine oxidoreductase (XOR) activity.

The oxidation of xanthine to uric acid was assayed by monitoring the increase in absorbance at 295 nm with the electron acceptors NAD^+ and O_2 (Waud & Rajagopalan, 1976). The reaction was carried out with 100 μl volume as summarized in table 19. The specific activity was calculated using the molar extinction coefficient for uric acid, which is $9,600 \text{ M}^{-1}\text{cm}^{-1}$.

Table 19: The assay set used for XOR activity in *D. melanogaster*.

Example of XDH activity assay			Example of the XDH blank		
Reagent	Volume	Final concentration	Reagent	Volume	Final concentration
1.5 mM xanthine in 10 mM NaOH	10 μl	0.15 mM	10 mM NaOH	10 μl	1 mM
100 mM Tris HCL, pH 7.5	20 μl	20 mM	100 mM Tris HCL, pH 8	20 μl	20 mM
6.7 mM NAD^+	10 μl	0.67 mM	6.7 mM NAD^+	10 μl	0.67 mM
<i>Drosophila</i> gel-filtered cytosol	60 μl		<i>Drosophila</i> gel-filtered cytosol	60 μl	

XDH activity was calculated by subtracting the blank measurements from the XDH assay.

2.7.5. Determination of specific activity.

Specific activity is expressed as the number of nmoles of substrate transformed per min per mg of protein at incubation temperature. The number of nmoles of substrate biotransformed per min was then calculated from the absorbance change observed per min using the relationship:

$c = A/\epsilon l$ where c = concentration (mM), A = absorbance, l = path length of absorbing solution in cm, ϵ = molar extinction coefficient. The light path for 100 μl in a microplate was 0.681cm.

2.8. Determination of optimum conditions of molybdo-flavo enzymes.

As the rate of enzyme activity may be changed according to the reaction conditions (pH, temperature and substrate concentration) that affect the activity of molybdo-flavo enzymes these were investigated.

2.8.1. Determination of optimum pH of buffer.

The pH stability of AO and XDH were analyzed by incubation at 37°C for 5 min at different pH values 5.6, 6.5, 7, 7.5 and 8 using sodium phosphate buffer (50 mM final concentration). The assay was carried out at constant substrate 0.1mM final concentration for DMAC and 0.15mM final concentration for xanthine and the cytosol volume 40 µl for the DMAC oxidase assay and 60 µl for the XDH assay.

2.8.2. Temperature dependence of AO and XDH activities in *Drosophila* extracts.

The activities of AO and XDH were measured at different temperature. The aliquots of filtered cytosol were incubated at 20, 25, 30, 37, 45 and 50°C for 30 min in a thermocycler machine (PCR machine) with a heated lid to prevented losses due to refluxing. After incubation the cytosols were removed from heating and cooled in ice for 5 min. The cooled cytosol extracts were then centrifuged at 13,000 xg for 5 min and the precipitate discarded. The assays was carried out at 0.1 mM final substrate concentration of DMAC for AO activity and 0.15 mM final concentration of xanthine for XDH activity, as described in tables 16 and 19.

2.8.3. Determination kinetic parameters (K_m) and maximum velocity (V_{max}) for molybdo-flavo enzymes enzyme at different substrate concentrations.

The rate of oxidation of varying concentrations of substrate of AO and XDH were monitored over the first two minutes of reactions. The enzyme was diluted to provide suitable reaction rates over an appropriate range of substrate concentrations were used. The substrates concentrations in the assay were 25 – 100 µM for the AO substrates, DMAC, vanillin and phenanthridine and XDH substrate, xanthine. Typically 40 µl of

cytosol solution was added to each assay. The initial velocity V , corresponding to each substrate concentrations $[S]$ was determined by measurement of the gradient of the line recorded on the curve and expressed as the change in absorbance/time. An Eadie Hofstee $V/[S]$ versus $[S]$ was then plotted (Figure 27). The intercept on the ordinate axis is V_{\max} ; the slope is K_m . The line of best fit through the points on plot was determined using linear regression by least squares method using Microsoft Excel (Microsoft Office). All determinations were carried out using BioTek microplate reader at 37°C.

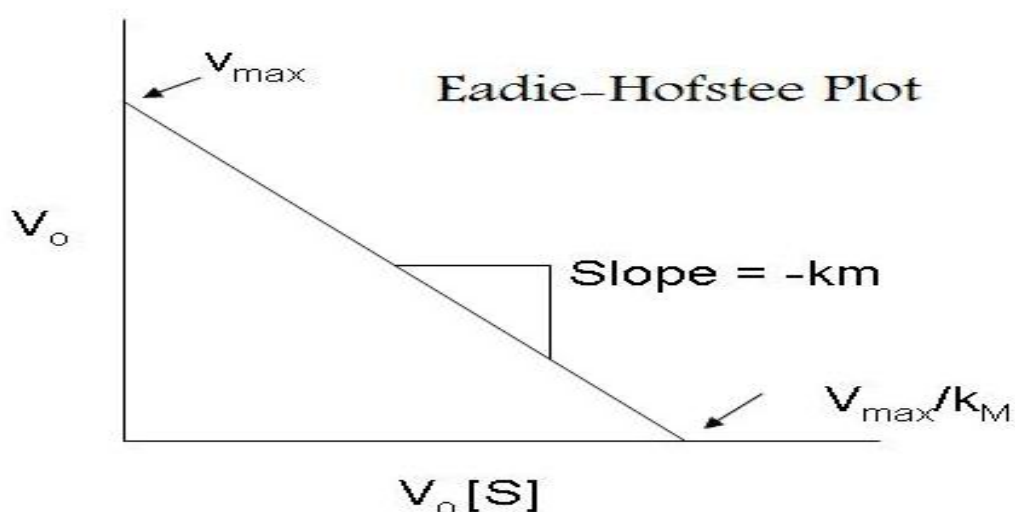


Figure 27: A plot of the Eadie- Hofstee.

The plot was used to define the V_{\max} of the enzyme-catalysed reaction and the K_m constant for the AO and XDH enzymes.

2.9. DNA analysis

2.9.1 Bioinformatics.

Alignment of sequences and primer design was performed using GeneJockey™ (Biosoft, Cambridge). In some cases primers had to be manually designed and checked for primer dimer and hairpin loop formation using Amplify®, a freeware program obtained from Bill Engles, Department of Genetics, University of Wisconsin, USA. Both programs were run on an Apple Macintosh computer. DNA sequences were obtained from the National Center for Bioinformatics website (NCBI) situated at (<http://www.ncbi.nlm.nih.gov/>). After the primers were designed a BLAST alignment was carried out online at (<http://www.ncbi.nlm.nih.gov/>), this program aligns the inputted sequences with all of the sequences found on the NCBI database. Primers were discarded if they were found to bind to the target gene in more than one place, or if they bound to other genes found in the *Drosophila* genome. DNA sequences were translated using the genetic code as shown in appendix 1. The one letter and three letter amino acid codes are also listed in appendix 2.

2.9.2. Extraction of DNA from *Drosophila melanogaster*.

This was carried out using a Qiagen Dneasy™ Tissue Kit according to the manufacturer's instructions:

Drosophila melanogaster strains were etherised for 60 seconds leading to death. 70 mg of *Drosophila melanogaster* was then homogenised in 180 µl phosphate buffer saline (PBS) by using disposable plastic homogeniser to crush the flies. The 1.5 ml Eppendorf tubes containing the flies were then balanced and centrifuged at 10000 rpm for 5 minutes. The supernatants were then cautiously transferred using a pipette with autoclaved tips. The supernatants were then transferred into new and labelled eppendorf tubes. The centrifugation process was then repeated. About 130µl of the supernatants were carefully removed and put into new labelled 1.5 ml eppendorf

tubes. Care was taken when the supernatants were removed to avoid getting some of the precipitants in them. The new fractions were then used for DNA extraction. 20 µl proteinase K and 200 µl buffer AL was added to the supernatants which were then incubated at 70°C for 10 minutes. 200 µl ethanol was then added to the samples which were vortex mixed and the mixture pipetted into DNeasy spin column. Samples were then centrifuged at 6000 rpm for 1 minute. 500 µl buffer AW1 was then added and the sample centrifuged for 1 minute at 6000 rpm. 500 µl of AW2 of buffer was added then and the sample was centrifuged at 13000 rpm for 3 minutes, the flow-through was then discarded. The DNeasy spin column was then placed in a new collection tube and the DNA was eluted in 200 µl buffer AE with centrifugation at 6000 rpm for 1 minute. For storage the DNA solution was placed at - 70.

2.9.3. Polymerase chain reaction cloning of molybdenum cofactor sulfurase gene.

The following materials and methods were used in the cloning process for the *Drosophila melanogaster* molybdenum cofactor sulfurase gene.

2.9.4. Polymerase chain reaction.

Polymerase chain reaction (PCR) reagents were from Promega, UK and it consisted of 25mM MgCl₂, dNTPs, PCR buffer, and Taq DNA polymerase. Distilled autoclaved water was used for dilution. Disposable tips, 1.5ml eppendorf and 0.5 ml PCR tubes were used with appropriate labelling. All pipetting steps were carried out in a sterile environment, and all the pipette tips and tubes used were autoclaved before use. All reagents were vortexed before use and all samples vortexed after being pipetted. The dNTPs mix, PCR buffer, primer solutions, 25mM MgCl₂ and *Drosophila* strain samples were thawed before use, and kept on ice. The following (Table 20) shows the primers used in the PCR of the molybdenum cofactor sulfurase gene.

Table 20: Summary of the primers designed for the PCR cloning of the *Drosophila* molybdenum cofactor sulfurase gene. Nucleotide sequences and localization of the primers designed for the PCR amplification of *Drosophila* MCS gene.

Primer Code	Primer Sequence* 5'-3'	bp at which primers start and finish**	Expected Product Size	Code for PCR product
DMCS1F	TATTGGTGAGCAGTGTG	-323	575 bp	<i>mal-1</i>
DMCS1R	ACACGGATGAACTCACTGAATCG	+898		
DMCS2F	TTCCAGCGAAGAAGAGTATG	+495	732 bp	<i>mal-2</i>
DMCS2R	AGCACTGAGGTGTGGTTCTC	+1227		
DMCS3F	AGCCGAGGTCTTCCAGAA	+1642	780 bp	<i>mal-3</i>
DMCS3R	TTCAGCAACGGCGGCAAT	+2422		
DMCS4F	AGCCGAGGTCTTCCAGAA	+1642	1103 bp	<i>mal-4</i>
DMCS4R	GTCGGCACACCTTGCTTACGCATT	+2745		
DMCS5F	CTGCCATTCTCTCCATTGTCTG	+1760	662 bp	<i>mal-5</i>
DMCS5R	TTCAGCAACGGCGGCAAT	+2422		
DMCS6F	CTGCCATTCTCTCCATTGTCTG	+1760	985 bp	<i>mal-6</i>
DMC6R	GTCGGCACACCTTGCTTACGCATT	+2745		
DMCS7F	AATACTCCTGCGGACTGG	+2126	615 bp	<i>mal-7</i>
DMCS7R	GCACACCTTGCTTACGCA	+2741		
DMCS8F	GACAACTATTCAGGACG	+2300	447 bp	<i>mal-8</i>
DMC8R	CTGTCGGCACACCTTGCTTA	+2747		
DMCS9F	GACAACTATTCAGGACG	+2300	883 bp	<i>mal-9</i>
DMCS9R	TAGTGATGTAGATGCCGAATCGCA	+3183		

*reading 5'-3' ** bp at which the 5' base of the primer binds in relation to the A of the initiator codon being +1.

2.9.5. The PCR protocol.

The PCR protocol was as outlined in the table below. All reactions are run for 35 cycles in total volume 50 µl.

Table 21: Summary of the volumes and concentrations of each component used in PCR reaction of the molybdenum sulfurase cofactor gene.

Component	Final Volume	Final concentration
Autoclaved distilled water	30-33µL	-
5X Green GoTaq® Flexi Buffer (Promega.UK).	10µL	1x
MgCl ₂ Solution (25 mM; Promega.UK)	3-6µL	1.5-3mM
dNTP Mix (10 mM each nucleotide)	1µL	200 µM (each nucleotide)
Primer mix (50 pmoles/µL each primer forward and reverse; MWG Biotech, Ebersberg, Germany)	0.5µL	0.1 µM (each primer)
Go Taq® DNA polymerase (5U/µl); Promega.UK)	0.5µL	2.5 Unit/50µL
Genomic DNA template	1.0µL	~ 0.5 µg/50µL

A typical PCR reaction was carried out using the following conditions:

Cycle 1

1. Denature 94°C for 3 minutes
2. Anneal 42-65°C for 1 minute
3. Extend 72°C for 1 minute/kb

Cycles 2-35

1. Denature 94°C for 1 minute
2. Anneal 45-67°C for 1 minute
3. Extend 72°C for 5 minutes

The number of cycles were 35 cycle and the whole PCR procedure took approximately 2 hr and 30 min. The success of the PCR was evaluated by agarose gel electrophoresis.

2.9.6. Agarose gel electrophoresis.

Agarose gel electrophoresis was carried out according to the methods described by Maniatis *et al.*, 1982. For a 1% w/v agarose gel, 1 g of agarose was added to 100 ml 0.5 X TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA pH 8). This mixture was then heated in a microwave oven until all the agarose was dissolved. The heated mixture was allowed to cool to 60°C and 1µl of 10mg/ml ethidium bromide was added. This was poured into a 11 x 14 cm gel tray, and a 2 mm wide well former inserted and the gel allowed to set for approximately 30 minutes. The gel was then placed into a horizontal gel electrophoresis apparatus with the wells at the anode side. 0.5 X TBE was then poured into the tank until the gel was submerged to a depth of 2-3 mm. For reactions containing the 5X Green GoTaq® Flexi Buffer, the reaction was loaded directly onto the gel after amplification. In order to approximate the size of the PCR products 4 µl of 1 kb DNA ladder purchased from Promega (Southampton, UK.) was mixed with 1 µl loading dye. This 1 kb ladder produced 13 fragments of 250 bp, 500 bp, 750 bp, 1,000 bp, 2,000 bp, 2,500bp, 3,000 bp, 4,000 bp, 5,000 bp, 6,000 bp,

8,000 bp and 10,000 bp. The PCR products or size markers were loaded into individual wells of the gel and electrophoresed for 1 hour and 15 minutes at 100 V, 200 mA. The gels were then visualised on an UV transilluminator with wavelength 254 nm and photographs taken as a record.

2.9.7. Preparation of PCR samples for sequencing.

The PCR samples were prepared for sequencing using the QIAquickTM purification kit (Qiagen; UK). All centrifugation steps were carried out in a benchtop microcentrifuge at 13000 rpm. The method followed manufacturers instructions as follows:

200 µl of buffer PB were added to 40 µl PCR products and mixed. This was then applied to the QIAquickTM column and centrifuged for 60 seconds. The flow through was discarded and 750 µl of buffer PE added to the column. This was centrifuged for 60 seconds and the flow through discarded then centrifuged again for another 60 seconds. Finally the QIAquickTM column was placed into a clean tube and 50 µl buffer EB applied to the centre of the membrane and centrifuged for 60 seconds to elute the DNA. Once the PCR product has been purified into a sterile 1.5 ml tube then appropriate reverse and forward primers were used to sequence the purified PCR products. 10 pmol/µl stock of the reverse or forward primer was prepared by pipetting 10 µl of primer 100 pmol/ µl stock and 90µl of sterile distilled water into an Eppendorf tube and vortexed. 1.5µl of a single 10 pmol/ µl primer stock was then and put into a sterile tube containing each of the purified PCR samples (40 µl) for reverse and forward direction sequencing. All purified samples were stored at -20°C until required for sequencing.

2.10. Statistical analysis.

2.10.1. Chi² Statistical Tests.

Using a Chi² statistical test (χ^2), it was determined whether or not the frequency distribution of the collected data was as a result of a something occurring by chance.

Using the formula:

$$\chi^2 = \sum (\text{Observed value} - \text{Expected value})^2 / \text{Expected value}.$$

The expected value was calculated as follows:

$$E = (\text{Total of column} \times \text{Total of row}) / \text{Overall total}.$$

The degree of freedom (df) was calculated as follows:

$$df = (\text{Number of rows} - 1) \times (\text{Number of columns} - 1).$$

For these results, the critical values (5% significance or $P < 0.05$) for χ^2 were 3.84, 5.99, and 7.82, since there were 1, 2, and 3 degrees of freedom respectively. The null hypothesis (H_0) was accepted if the calculated value was less than the critical value, while H_0 was rejected and the alternative hypothesis (H_1) accepted if the calculated value was more than the critical value. A Yates correction value of 0.5 was used when the df was 1. H_0 states that there is no significant difference between the observed and expected values, while H_1 states that there is a significant difference between the observed and expected values.

2.10.2. T-test two tailed.

The results were calculated as the mean \pm standard deviation (SD) for the number of experiments. Statistical significance between samples group were compared by a t-test two tailed. Values with $P < 0.05$ were considered statistically significant. The t- test is the parametric test for calculating the significance of a small sample mean because the distributions of small sample are not normal.

A one sample t-test has the following null hypothesis:

$$H_0 : \mu = c \quad \mu \text{ (mu) = the population mean, } c = \text{hypothesized value.}$$

3. Results.

This results section is split into 6 main sections as follows. Firstly sections 3.1 and 3.2 spectrophotometric and HPLC assays were used to assess molybdo-flavoenzymes mediated biotransformation in wild type, transgenic and MFEs deficient *Drosophila*. Toxicological and metabolite assays are then described to determine the chemoprotective role of AO and XOR in wild type and genetically deficient *Drosophila melanogaster* in sections 3.3. The effect of MFEs deficiencies on lifespan and the effects of cold and heat shock were studied as described in sections 3.4 and 3.5. Finally, the cloning and DNA sequencing of the molybdenum cofactor sulphurase (MCS) gene in wild type and mutant strains are subsequently described in section 3.6.

3.1. Quantification of molybdo-flavoenzyme activity in wild type and mutant *Drosophila melanogaster* strains.

3.1.1. Investigation of molybdo-flavoenzyme activities in Canton-S *Drosophila* strains using spectrophotometric assays.

Several spectrophotometric assays were used to measure the relative reaction rates of the molybdo-flavoenzymes to be studied. These assays were designed to specifically measure the rate of biotransformation of one N-heterocyclic AO substrate (phenanthridine) and two aldehyde compounds (vanillin and dimethylaminocinnamaldehyde (DMAC)). XOR was measured with or without NAD⁺ in the assay using its endogenous substrate xanthine. Assays were performed at 37°C as described in materials and methods section 2.7 with 50 µl of Canton-S cytosol and 0.1 mM final substrate concentration. In order to remove endogenous inhibitors cytosol was gel filtered on a Sephadex G25 column prior to XOR assays. DMAC and vanillin oxidase assays monitored substrate disappearance at 398 and 310 nm respectively (see Figure 28a and 28b). Phenanthridine oxidase and XOR were monitored by following product formation of 6(5H)-phenanthridone and uric acid at

322 and 295 nm respectively (Figure 28c and 28d). All assays showed linear reaction kinetics under the conditions used. Omission of NAD^+ from the XOR assay resulted in no detectable production of uric acid reflecting the previously documented lack of activity of invertebrate XOR with oxygen as electron acceptor (Garattini *et al.*, 2003).

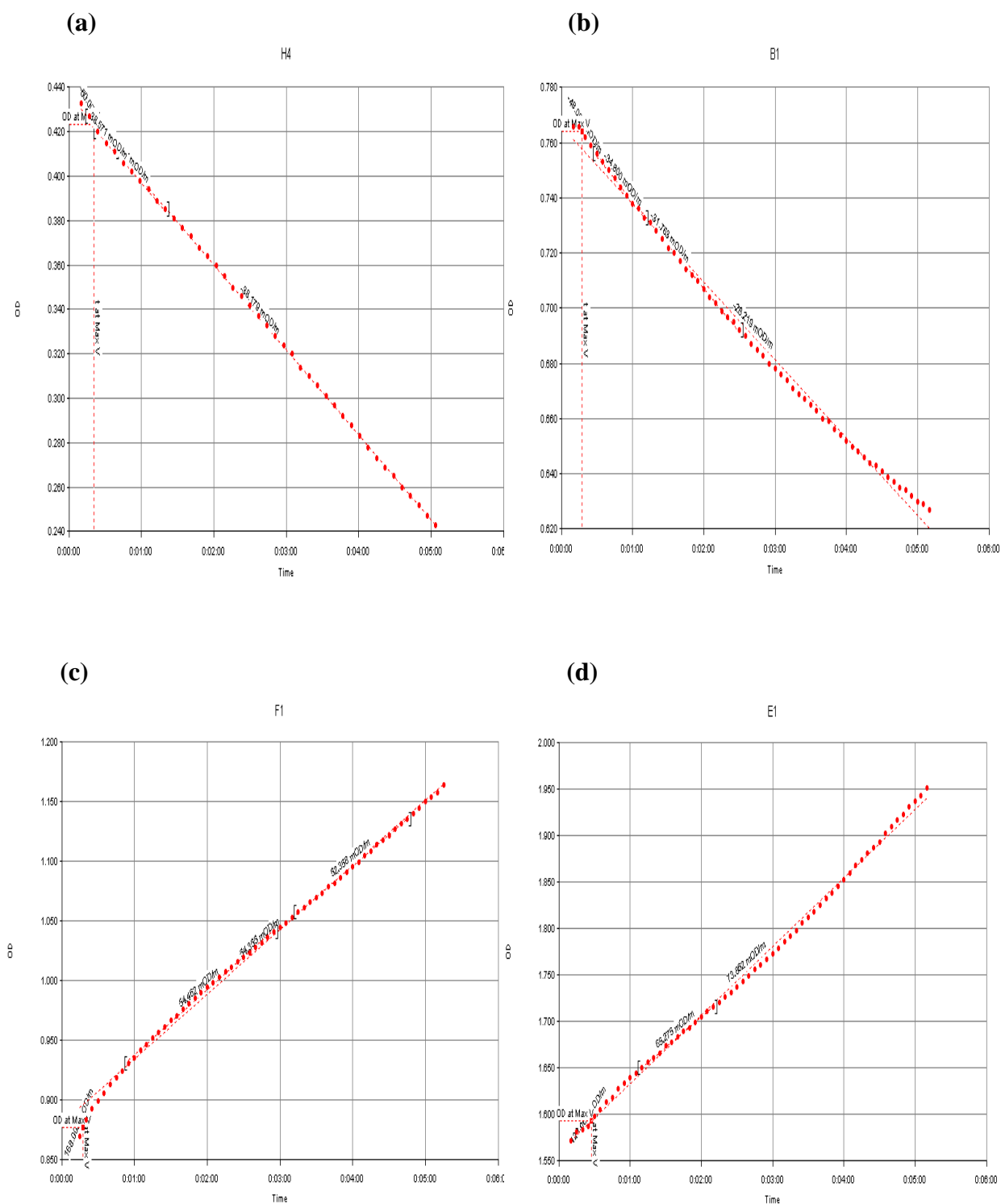


Figure 28: Spectrophotometric assay of aldehyde oxidase and xanthine dehydrogenase activities using Canton-S strain cytosol at 37°C with AO and XDH substrates.

(a) DMAC oxidase activity assay. (b) Vanillin oxidase activity assay. (c) Phenanthridine oxidase activity assay. (d) Xanthine oxidase activity assay. For all assays 100 μM final substrate concentrations were used. For the XDH assay 670 μM NAD^+ was used as the electron acceptor. For analysis conditions see section 2.7.

3.1.2. Effect of pH and temperature on *Drosophila* molybdo-flavoenzymes activity

In the initial assays 37°C was used for assays but in order to determine if this was the optimum temperature to use a series of experiments were performed. In order to test effect of temperature on the activity of *Drosophila* AO and XDH this was measured as at different incubation temperatures. The rate of reaction of *Drosophila* AO with DMAC rose gradually with increasing temperature from 20°C to 37°C, then fell to zero at 45°C as shown in figure 31. In contrast *Drosophila* XDH had approximately 80% of the activity at 45°C versus 37°C (Figure 29). As the maximum reaction rate was obtained at 37°C for both enzymes, this temperature was therefore selected for all assays.

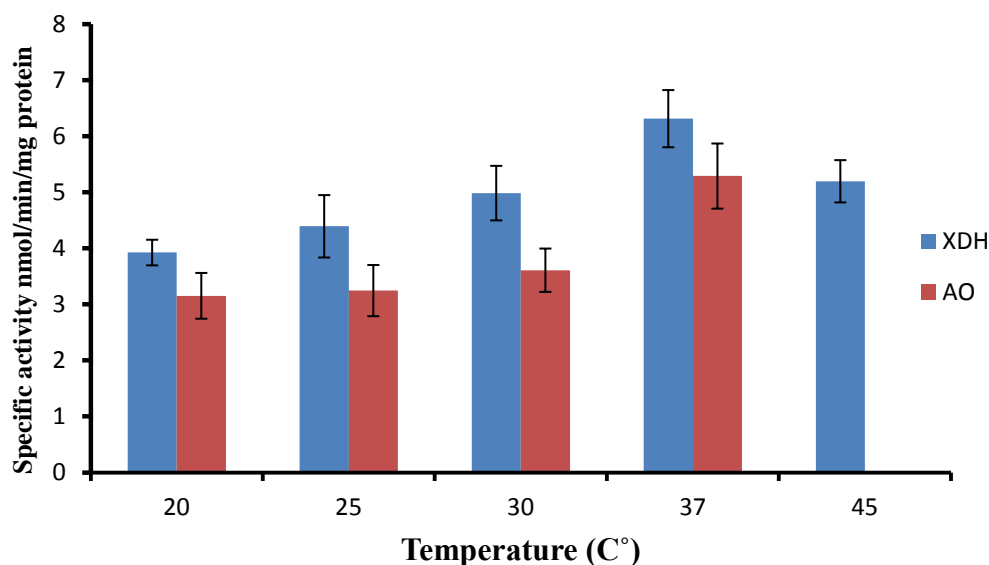


Figure 29: Effect of the assay incubation temperature on of Canton-S strain AO and XDH activity.

Enzyme activity was measured at 37°C, pH 7.5. 0.1 mM DMAC was used as the substrate in AO assays. 0.15 mM xanthine and 0.67 mM NAD⁺ were in XOR substrate assays. Each point represents the mean \pm S.D. of three experiments with pooled cytosol from ~1000 animals.

In order to test the effect of pH on *Drosophila* AO and XDH these were assayed under standard assay condition, except that the reaction pH was varied between 5.6

and 8.0 as described in section 2.8.1. The maximum activity of AO and XDH were found between pH 7 and 7.5 as shown figure 30.

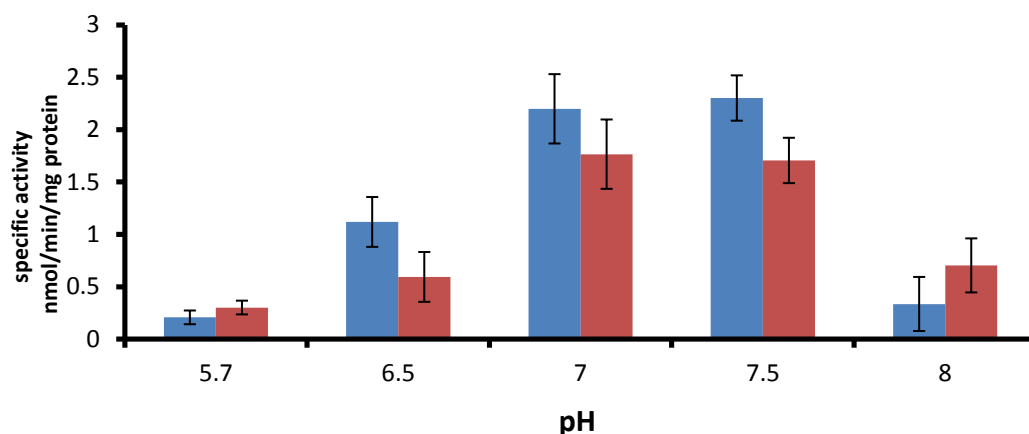


Figure 30: Effect of the pH on Canton-S strain AO (blue line) and XDH activity (red line).

AO activity was measured at 37°C. 0.1 mM DMAC was used as the substrate in AO assay. 0.15 mM xanthine and 0.67 mM NAD⁺ was used as a substrate XOR substrate assays. Each point represents the mean \pm S.D. of three experiments with pooled cytosol from ~1000 animals.

3.1.3. Comparison of MFE activity in wild type and mutant *Drosophila melanogaster* strains

Once the assays were optimised, AO and XDH activities were measured in the wild type and mutant strains. The results from the DMAC, vanillin and phenanthridine oxidase assays were collated from all *Drosophila* strains and are summarised in the following table 22. Both Canton-S and *ry*⁵⁰⁶ had activity towards all three AO substrates tested. The results demonstrate the significantly higher ($P < 0.05$) vanillin oxidase activity which is present in the rosy strains in comparison to Canton-S strain tested (Table 22). In contrast *mal-1* and *mal-f1* showed no DMAC, vanillin and phenanthridine oxidase activity. XOR activity was absent in *ry*⁵⁰⁶ and maroon-like strains. In order to determine that there was definitely no activity present in maroon-like strains for phenanthridine and vanillin as substrates a HPLC assay was performed

with these substrates. This is shown in figure 31, where no product was detected even after extended incubation periods. Similarly no uric acid production was detected using a HPLC assay with both *ry*⁵⁰⁶ and *mal-l* strains (Figure 32).

Table 22: Dimethylaminocinnamaldehyde, phenanthridine, vanillin and xanthine oxidase activities in *Drosophila* cytosol of different strains.

<i>Drosophila</i> strains	DMAC oxidase activity (nmol/min/mg protein) (100 μ M)	Phenanthridine oxidase activity (nmol/min/mg protein) (100 μ M)	Vanillin oxidase activity (nmol/min/mg protein) (100 μ M)	Xanthine dehydrogenase activity (nmol/min/mg protein) (100 μ M)
Canton-S	1.18 \pm 0.06	0.98 \pm 0.06	1.68 \pm 0.06	1.21 \pm 0.09
<i>ry</i> ⁵⁰⁶	1.20 \pm 0.07	0.90 \pm 0.07	2.22* \pm 0.16	N.D.
<i>mal-l</i>	N.D.	N.D.	N.D.	N.D.
<i>mal-fl</i>	N.D.	N.D.	N.D.	N.D.

Activities were determined as described in materials and methods (section 2.7.1). The activities are shown as nmol substrate hydroxylated/min/mg protein and are expressed as means \pm S.D. of 3 sets of pools of animals. The result of pooled cytosol from ~ 1000 male flies. N.D. – not detectable; (**P* < 0.05 t-test two tailed are marked with asterisks), n = 3.

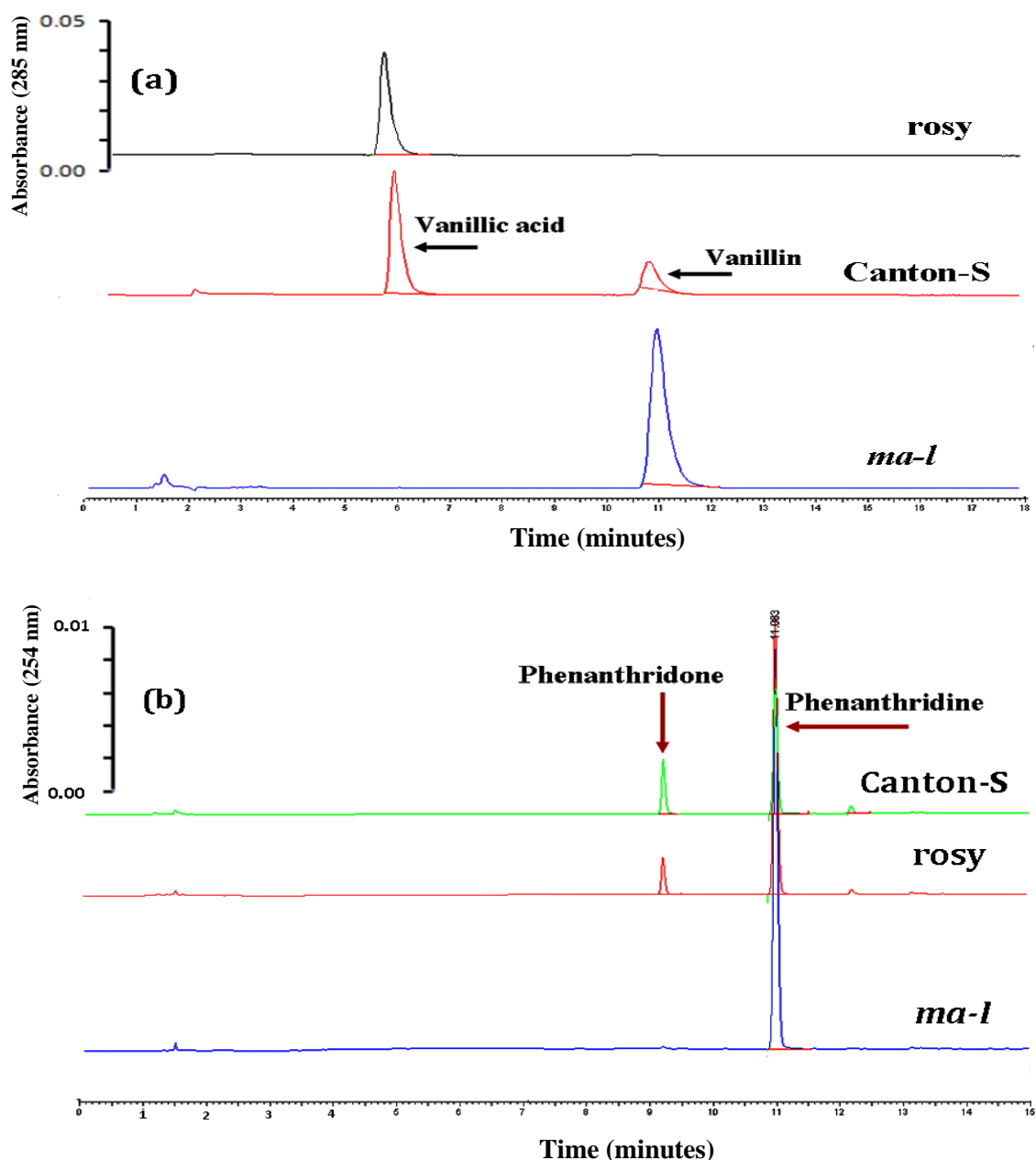


Figure 31: HPLC analysis of the *in vitro* biotransformation of vanillin and phenanthridine by Canton-S, rosy and maroon-like strains cytosol.

HPLC chromatograms are offset on the vertical axis to allow comparison between different *Drosophila* strains. (a) HPLC chromatogram of the *in vitro* oxidation of vanillin to vanillic acid by Canton-S, rosy and maroon-like cytosol after 20 minutes incubation time at 37°C. Lower HPLC chromatogram is *ma-l* strain. The middle chromatogram is Canton-S strain. The upper chromatogram shows oxidation of vanillin to vanillic acid for rosy strain. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.22 M KH_2PO_4 : acetonitrile system (85: 15) as mobile phase. The wavelength of the detection was 285 nm. (b) HPLC chromatogram the *in vitro* oxidation of phenanthridine by Canton-S, rosy and maroon-like cytosol after 30 minutes incubation time at 37°C. Lower HPLC chromatogram is *ma-l* strain. The middle chromatogram is Canton-S strain. The upper chromatogram is rosy strain. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with water: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 254 nm.

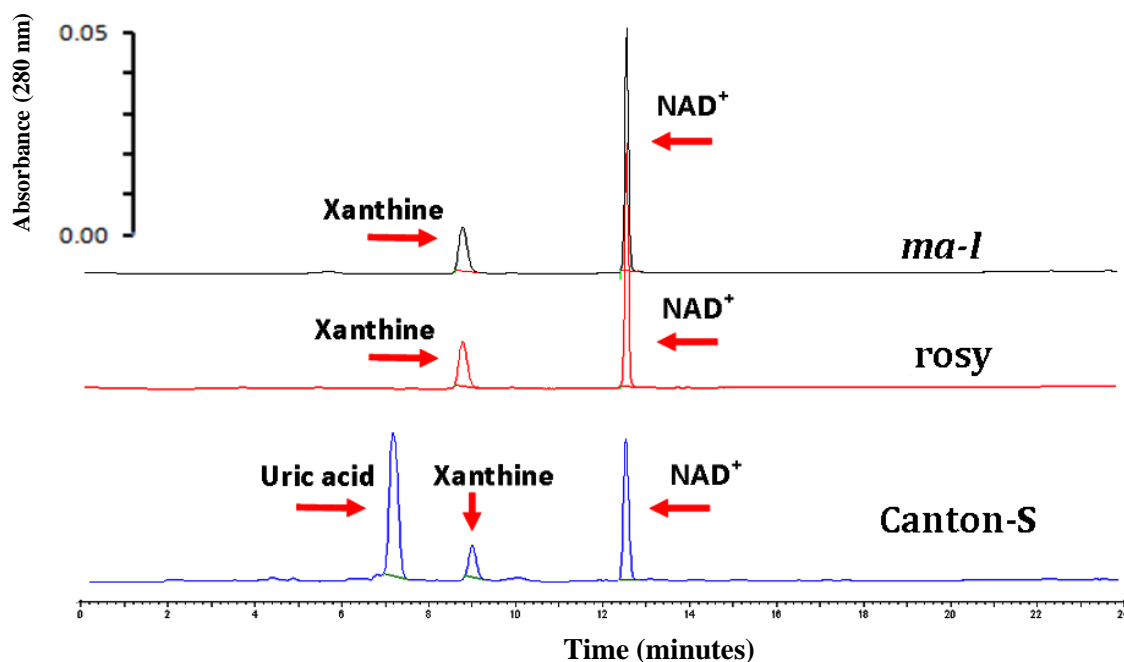


Figure 32: HPLC analysis of the *in vitro* biotransformation of xanthine by Canton-S, rosy and maroon-like cytosol after 120 minutes incubation time at 37°C.

Lower HPLC chromatogram is Canton-S strain. The middle chromatogram is rosy strain. The upper chromatogram is *ma-l* strain. HPLC chromatograms are offset on the vertical axis to allow comparison between different *Drosophila* strains. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

3.1.4. Spectrophotometric determination of the kinetic parameters for aldehyde oxidase and xanthine oxidoreductase in *Drosophila* strains.

Kinetic parameters of AO for the oxidation of DMAC, phenanthridine and vanillin were measured for AO in Canton-S and rosy strains. Mean values of K_m and V_{max} of AO and XDH were determined using Eadie Hofstee (Figures 33 and 34), due to this plot being superior to other methods of plotting data (Houston *et al.*, 2003). Eadie Hofstee plots of the data obtained gave good correlation coefficients (r^2) > 0.97 (Figures 33 and 34). The results obtained in the present study shows the at all substrates used were found to be good substrates of *Drosophila* AO (Table 23). K_m values of AO substrates were observed with DMAC (50 ± 5.4 μ M), phenanthridine (80 ± 9.1 μ M) and vanillin (303 ± 11.7 μ M) respectively for Canton-S strain cytosol.

Relatively low V_{\max} values were obtained with phenanthridine (1.78 ± 0.38 nmol/min/mg protein) and DMAC (1.80 ± 0.35 nmol/min/mg protein). The highest V_{\max} was obtained from Canton-S cytosol with vanillin (7.58 ± 2.11 nmol/min/mg protein) (Table 23). The enzyme kinetic parameters for AO and XDH are listed in table 23. Similar K_m and V_{\max} values were obtained for DMAC and phenanthridine as substrates for Canton-S and ry^{506} strains. K_m values for DMAC and phenanthridine were ~6 and ~4 fold lower than that for vanillin as a substrate. Interestingly vanillin oxidase activity in the ry^{506} strain was significantly different than the Canton-S strain. The K_m was approximately 15% lower and the V_{\max} was approximately 5% higher in the ry^{506} strain compared with the Canton-S strain (see figures 33 and 34).

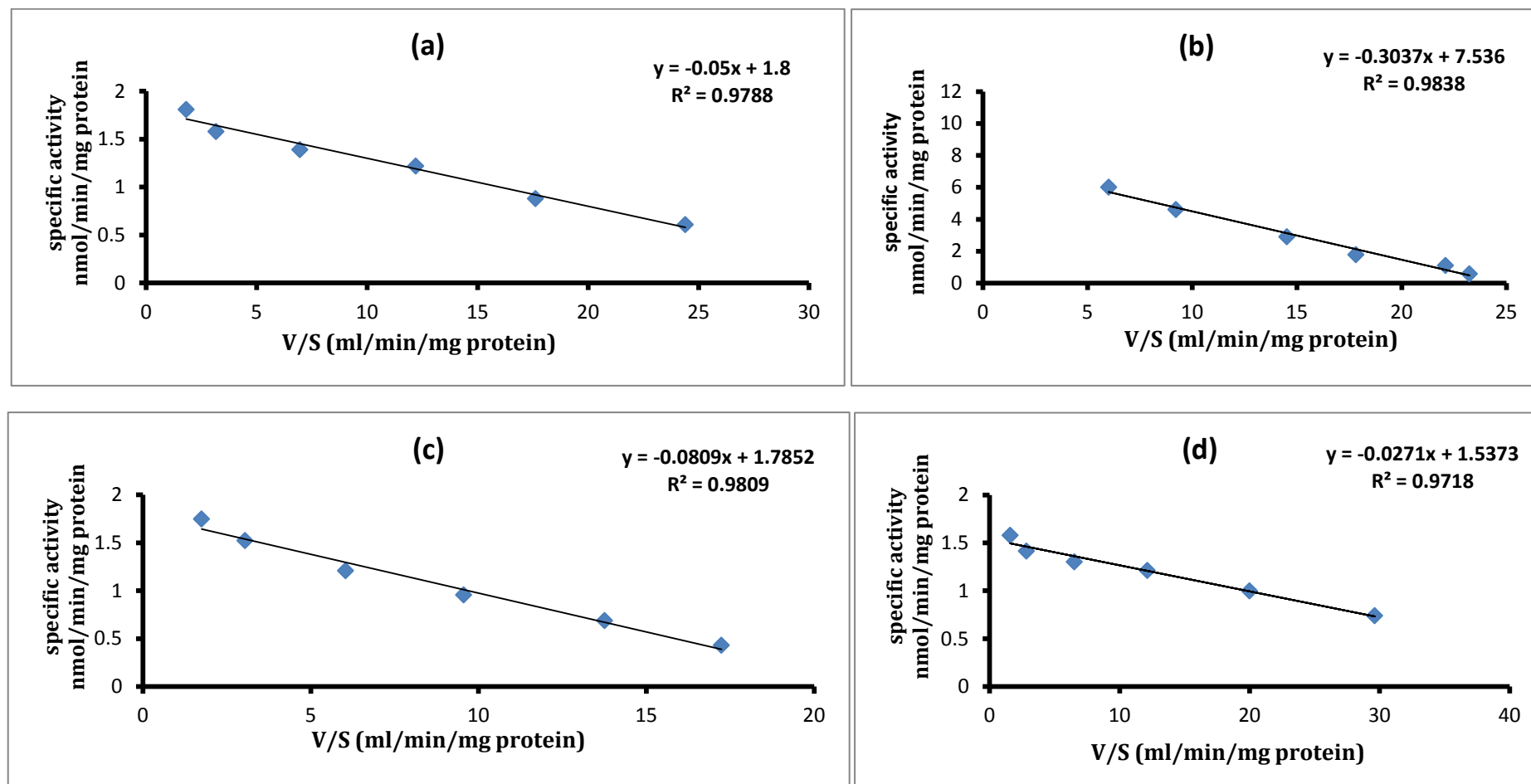


Figure 33: Eadie- Hofstee plot of AO and XDH activities in Canton-S *Drosophila* strain.

(a) AO-catalyzed oxidation DMAC. (b) AO-catalyzed oxidation vanillin. (c) AO-catalyzed oxidation phenanthridine. (d) XDH-catalyzed oxidation xanthine. The substrates concentrations were 25-100 μ M of AO and XDH substrates. The typical results are from three independent experiments.

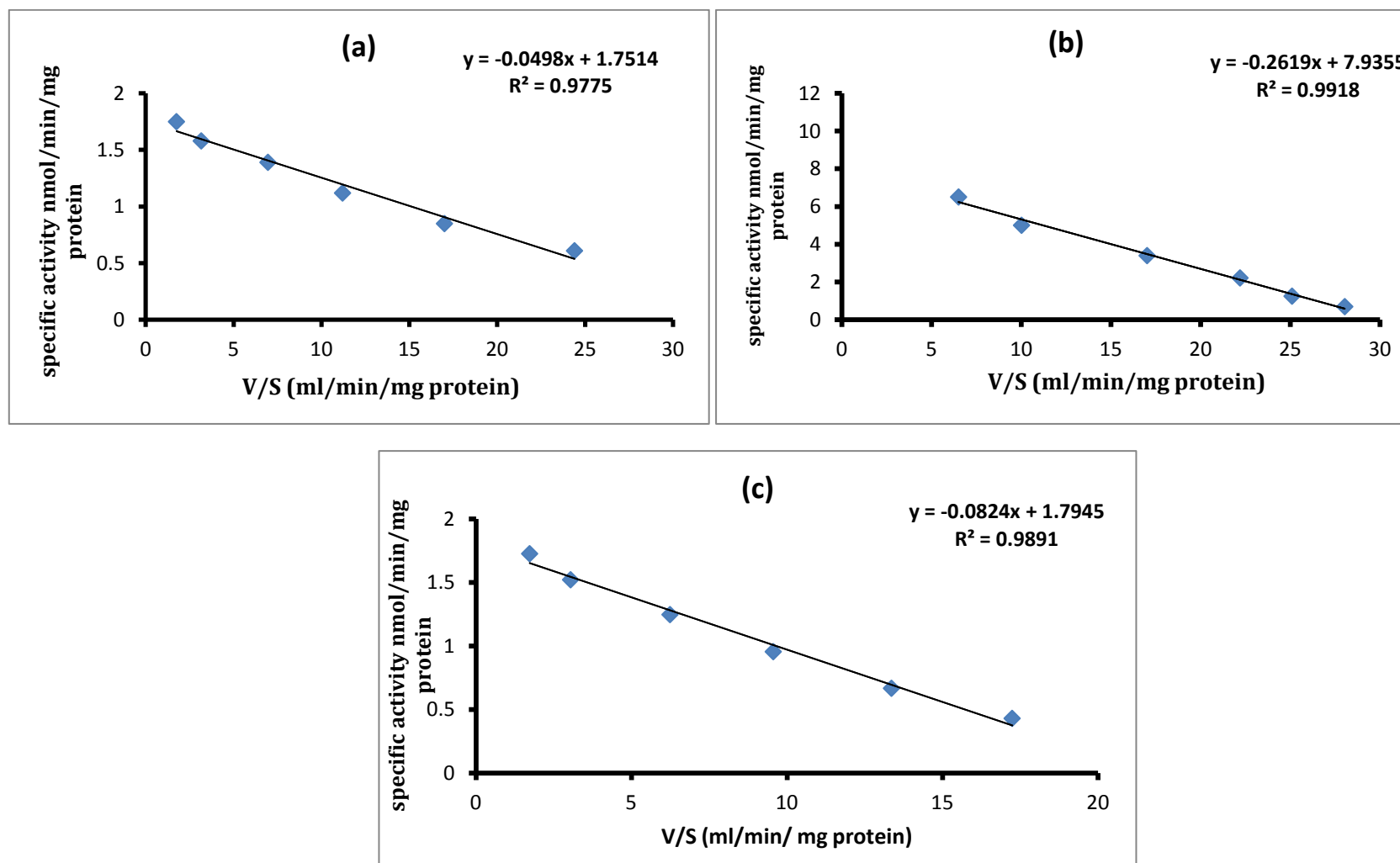


Figure 34: Eadie- Hofstee plot of AO activity in *ry*⁵⁰⁶ *Drosophila* strain.

(a) AO-catalyzed oxidation DMAC. (b) AO-catalyzed oxidation vanillin. (c) AO-catalyzed oxidation phenanthridine. The substrates concentrations were 25-100 μ M. The typical results are from three independent experiments.

Table 23: Kinetic constants for molybdo-flavoenzyme substrates by *Drosophila* strains cytosol.

Strains	substrates	K_m^{**} (μ M)	V_{max} nmol/min/mg protein
Canton-S	DMAC	50 \pm 5.4	1.80 \pm 0.35
	Phenanthridine	80 \pm 9.1	1.78 \pm 0.38
	Vanillin	303 \pm 11.7	7.58 \pm 2.11
	Xanthine	27 \pm 4.1	1.53 \pm 0.17
<i>ry</i> ⁵⁰⁶	DMAC	49 \pm 5.1	1.75 \pm 0.37
	Phenanthridine	82 \pm 9.3	1.79 \pm 0.42
	Vanillin	261* \pm 11.2	7.93 \pm 1.42
	Xanthine	N.D.	N.D.
<i>mal-1</i>	DMAC	N.D.	N.D.
	Phenanthridine	N.D.	N.D.
	Vanillin	N.D.	N.D.
	Xanthine	N.D.	N.D.
<i>mal-fl</i>	DMAC	N.D.	N.D.
	Phenanthridine	N.D.	N.D.
	Vanillin	N.D.	N.D.
	Xanthine	N.D.	N.D.

The oxidation rates of DMAC, phenanthridine and vanillin were measured spectrophotometrically at 37°C with molecular oxygen as an acceptor. The oxidation rate of xanthine by for xanthine dehydrogenase was measured spectrophotometrically at 37°C with NAD⁺ as an acceptor. K_m and V_{max} were calculated from linear oxidation rates using Eadie-Hofstee plots. All correlation coefficients (r^2) > 0.97. The value shown are the mean \pm S.D., obtained from three separate pooled cytosol fractions for each *Drosophila* strains, each experiment being conducted in triplicate. Substrates concentration = 100 μ M. The wavelength used for the DMAC, phenanthridine, vanillin and xanthine oxidase assays were 398, 322, 310 and 295 nm respectively. N.D. – not detectable; (* P < 0.05 t-test two tailed are marked with asterisks), **n = 3

3.2. Quantification of molybdo-flavoenzyme activities in wild type and mutant *Drosophila melanogaster* strains by HPLC assays.

As the majority of MFEs substrates and products cannot be quantified by spectrophotometry, HPLC assays were used to measure MFEs oxidation rates for 15 substrates.

3.2.1. Validation

3.2.1.1. Methods accuracy and precision of HPLC assays.

The precision of the various HPLC assays was determined by injecting five concentrations (0.01, 0.02, 0.03, 0.05 and 0.1 mM) of all AO and XOR substrates and the products listed in materials and methods section 2.5.2.2, three times on the same day to determine the intra-day variation. The intra-day variation assessed as the coefficient of variation in peak area ratio. The relative standard deviation (%RSD) calculation for retention time was described in section 2.5.2.2.1. The lower the value is the better the assay performance. At lower level it was below 3.5% for 3 replicates, see table 2 in appendix 3. The accuracy of the assay was calculated by comparison of the nominal analyte concentration to the actual concentration obtained from the linear regression line within the concentration range investigated (0.01 mM to 0.1 mM), see table 3 in appendix 3. Table 3 (in appendix 3) showed that the intra-day values ranged between 95.1-105.6%. The precision of all concentrations of analytes was approximately less than $\pm 5\%$. The calibration graphs obtained with these HPLC methods were linear over the concentration range used and the (r^2) values were 0.92 to 0.99 for each analyte when plotted for the mean peak area for three injections against the actual concentration (Figures 2-17 in appendix 3). This demonstrates that these HPLC methods are precise and repeatable and can be successfully used to analyze MFE catalysed biotransformations in this study.

3.2.1.2. Detection and quantification limits

In addition to accuracy and precision tests the limit of detection (LOD) and the limit of quantification (LOQ) were calculated for each of the analytes in section 2.5.2.3. The LOD of all substrates and products were ranged between 0.1-7 nmol and LOQ was ranged from 0.5 to 95 nmol. From these results, it can be concluded that the current HPLC methods are sufficiently sensitive to detect low concentrations of substrates and products of molybdo-flavoenzyme catalysed reactions enzymes. The results of linearity, limit of detection and limit of quantification are presented in table 4 in appendix 3.

3.2.2. HPLC analysis of the oxidation of purine based substrates by *Drosophila melanogaster* strains.

Prior to incubation with cytosol endogenous purines were removed by gel filtration using Sephadex G25 chromatography. The result obtained after incubation of Canton-S cytosol with xanthine as a substrate of XDH with and without NAD^+ present is shown on figure 35 a. No uric acid product was observed when NAD^+ was absent from the assays. In order to confirm that this was due to XDH the XDH inhibitor oxipurinol (0.1 mM final concentration) was added to the incubation (Figure 35 b). This shows that there was no uric acid generated and the reaction was completely inhibited by oxipurinol. Even using the sensitive HPLC assay no XOR activity with xanthine as a substrate was found for any of the MFE deficient strains (*ry*⁵⁰⁶, *mal-1* and *mal-f1*) (as shown in figure 32).

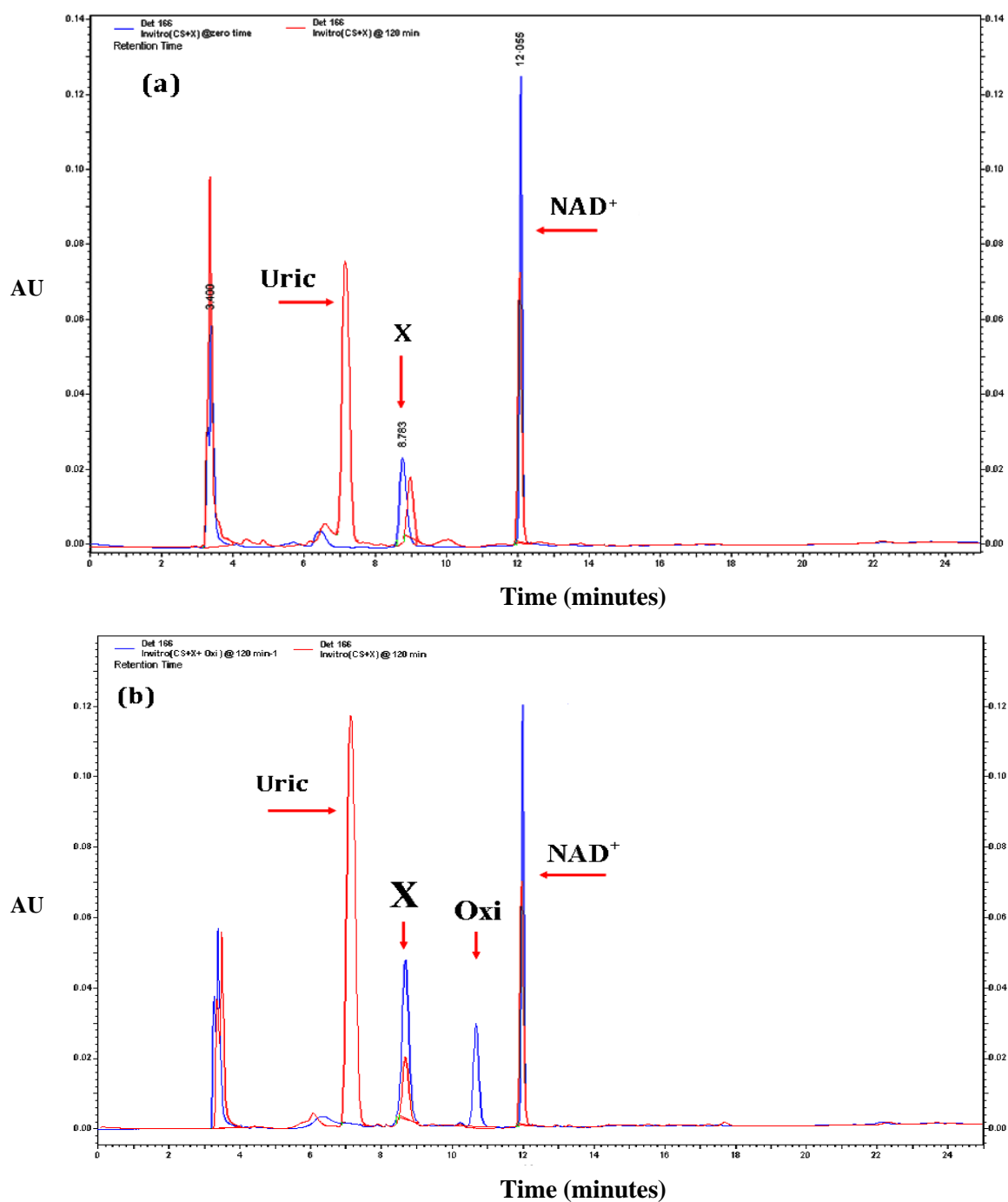


Figure 35: HPLC analysis of the *in vitro* biotransformation of xanthine by Canton-S cytosol.

(a) The chromatogram produced following zero and 120 minutes incubations are indicated by blue and red lines respectively. (b) HPLC analytes generated by Canton-S cytosol with xanthine and with and without oxipurinol. The chromatogram produced following 120 minutes with oxipurinol (blue line) and 120 minutes (red line) incubation without oxipurinol. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

With the major caffeine metabolite, 1-methylxanthine (1-MX) Canton-S cytosol shows that 1-methyl-uric acid (1-MU) was produced from 1-MX when NAD^+ was present as an electron acceptor (Figure 36). No 1-MU was produced when NAD^+ was absent (data not shown). 7.5 ± 1.4 nmol of 1-MU was produced/15 min/mg Canton-S cytosol. When similar assays were carried out with 3-methylxanthine (3-MX) and 7-methylxanthine (7-MX) no methyl-uric acids were generated even with extended time periods. This suggests that 3-MX and 7-MX were not substrates for *Drosophila* XDH.

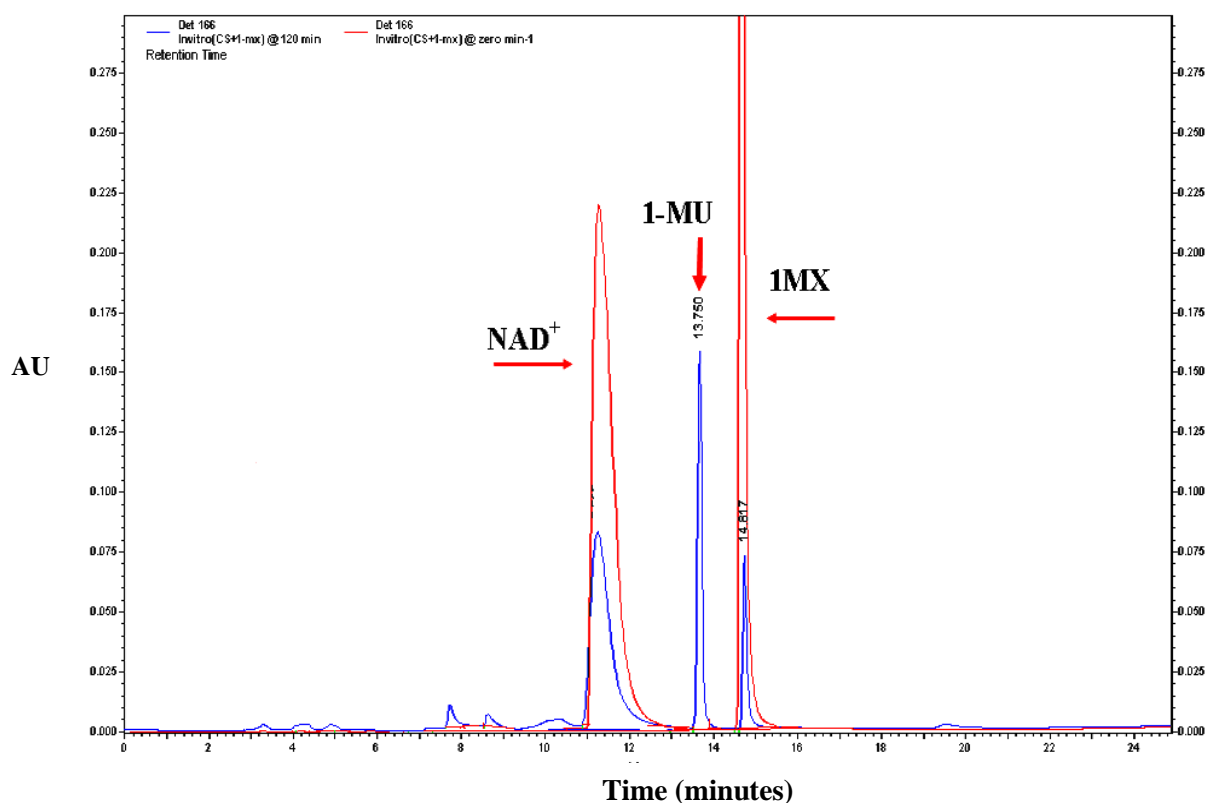


Figure 36: HPLC analysis of the *in vitro* biotransformation of 1-methylxanthine (1-MX) by Canton-S cytosol at 37°C.

The blue line is a HPLC chromatogram from 120 minutes incubation and red line is a HPLC chromatogram for a zero minute incubation. Analytes were injected onto a C-18 column (Kromasil 5 μm) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

6-Mercaptopurine (6-MP) is a thiopurine anti-leukemia drug in which carbon 6 of purine has been substituted with a sulphur atom (Merck Index, 2006). In mammals 6-MP is oxidized to 6-thiouric acid via 6-thioxanthine or 8-oxo-6-mercaptopurine by AO or XOR (Van Scoik *et al.*, 1985) although there is some controversy over the *in vitro* studies (Rashidi *et al.*, 2007; Rowland *et al.*, 1999). According to the *in vitro* experiments with *Drosophila* cytosol in this study, 6-MP is not a substrate for either XOR or AO of *Drosophila melanogaster* using NAD^+ and O_2 as electron acceptors respectively (shown figures 37 a and b). In order to validate that the assay, rabbit liver cytosol was incubated with 6-MP and the products generated analysed by HPLC. This demonstrated that when rabbit cytosol was incubated with 6-MP it was oxidized to 6-thiouric acid via 6-thioxanthine. This confirmed that the HPLC procedure and incubation conditions had successfully detected and resolved the expected 6-MP metabolites confirming the lack of detectable activity in *Drosophila* cytosol towards this substrate was not due to experimental conditions (Figure 38).

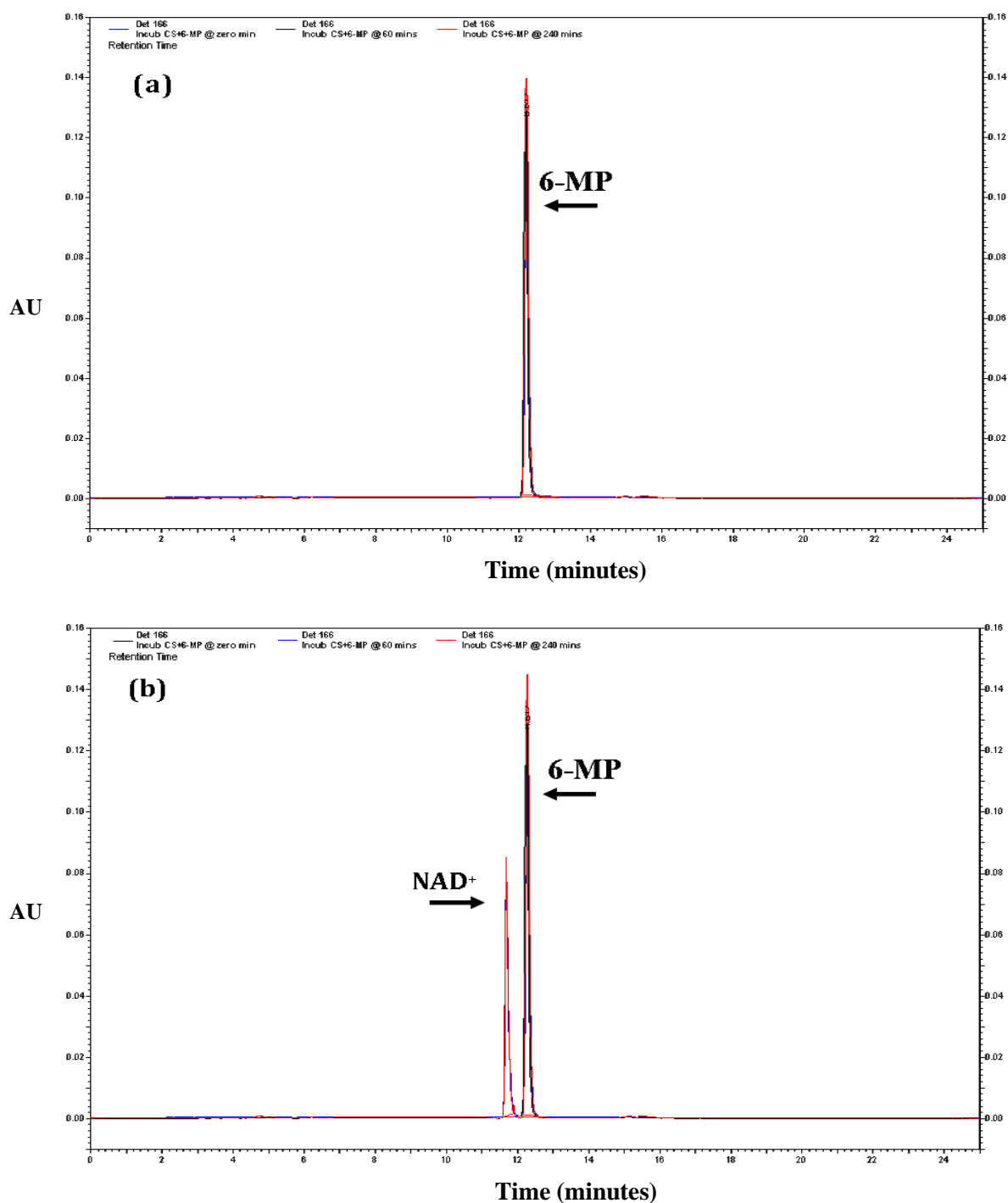


Figure 37: HPLC analysis of analytes produced following the *in vitro* incubation of 6-mercaptopurine with Canton-S cytosol with and without NAD⁺ at 37°C.

(a) HPLC chromatogram of analytes generated following incubation of 6-MP with Canton-S cytosol at 0, 60 and 240 minutes without NAD⁺. (b) HPLC chromatogram of analytes produced following the *in vitro* incubation of 6-MP with Canton-S cytosol at 0, 60 and 240 minutes with NAD⁺. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 355 nm.

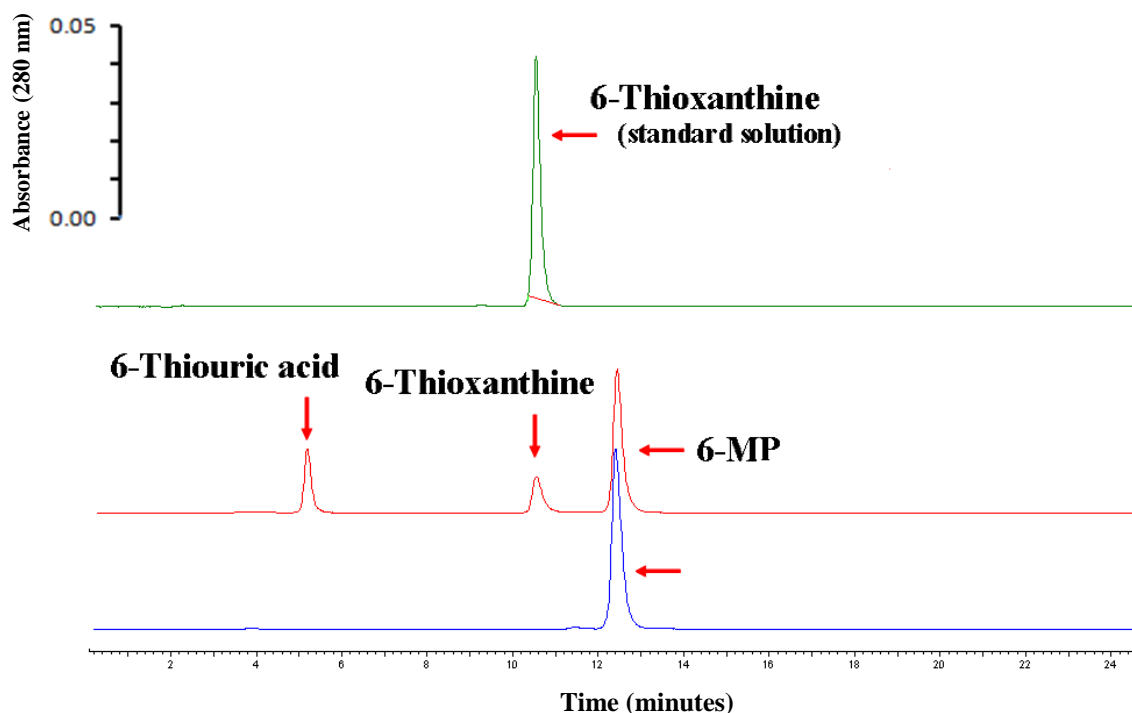


Figure 38: HPLC analysis of the *in vitro* biotransformation of 6-mercaptopurine by rabbit liver cytosol at 0 and 60 minutes.

Blue line is HPLC chromatograms of a zero minute incubation. Red line is HPLC chromatograms of 60 minutes incubation. The green line is 6-thioxanthine standard. HPLC chromatograms are offset on the vertical axis to allow comparison between incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μm) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

Allopurinol was also tested as a substrate with and without NAD^+ in the incubations with Canton-S strain cytosol. The results shown in figure 41 indicate that the product oxipurinol was formed with O_2 as an electron acceptor and that product formation was increased approximately 3 fold following NAD^+ addition (Figure 39 and Table 24).

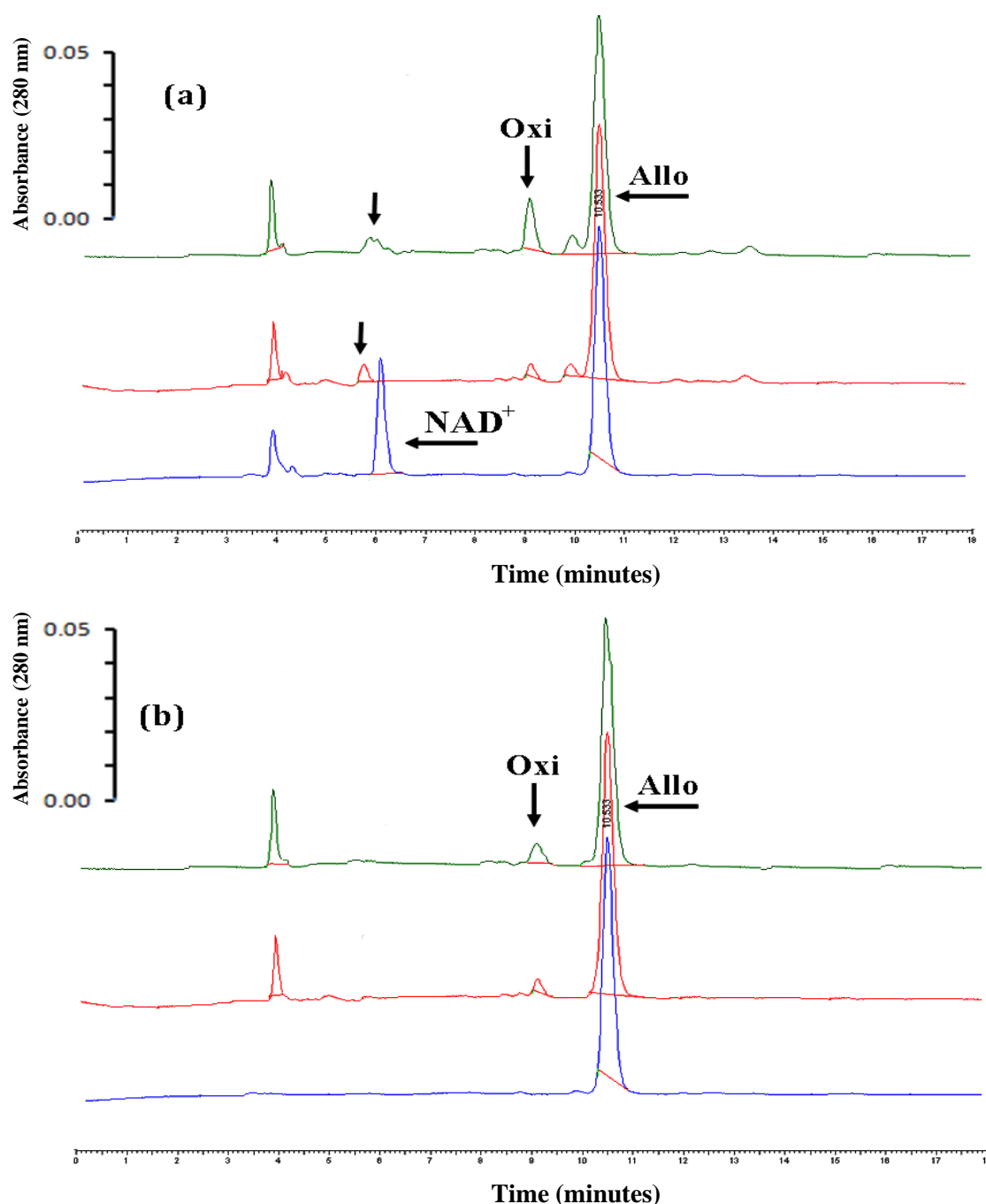


Figure 39: HPLC analysis of the *in vitro* biotransformation of allopurinol by Canton-S cytosol strain at 37°C with and without NAD⁺.

(a) Incubation with NAD⁺. Blue line is HPLC chromatograms from analytes from a zero minute incubation. Red and green lines are analytes from 30 and 120 minutes. (b) Incubation without NAD⁺. Blue line is HPLC chromatograms of a zero minute incubation. Red and green lines are 30 and 120 minutes. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

The chromatogram (Figure 40) shows that XOR-null rosy strain cytosol can also convert allopurinol to oxipurinol. In comparison, at the same incubation course for the rosy strain cytosol, there was a lower in the amount of product, when was compared with Canton-S strain. Using the sensitive HPLC assay no oxipurinol was produced with allopurinol as a substrate by MFE deficient strains cytosol (maroon-like).

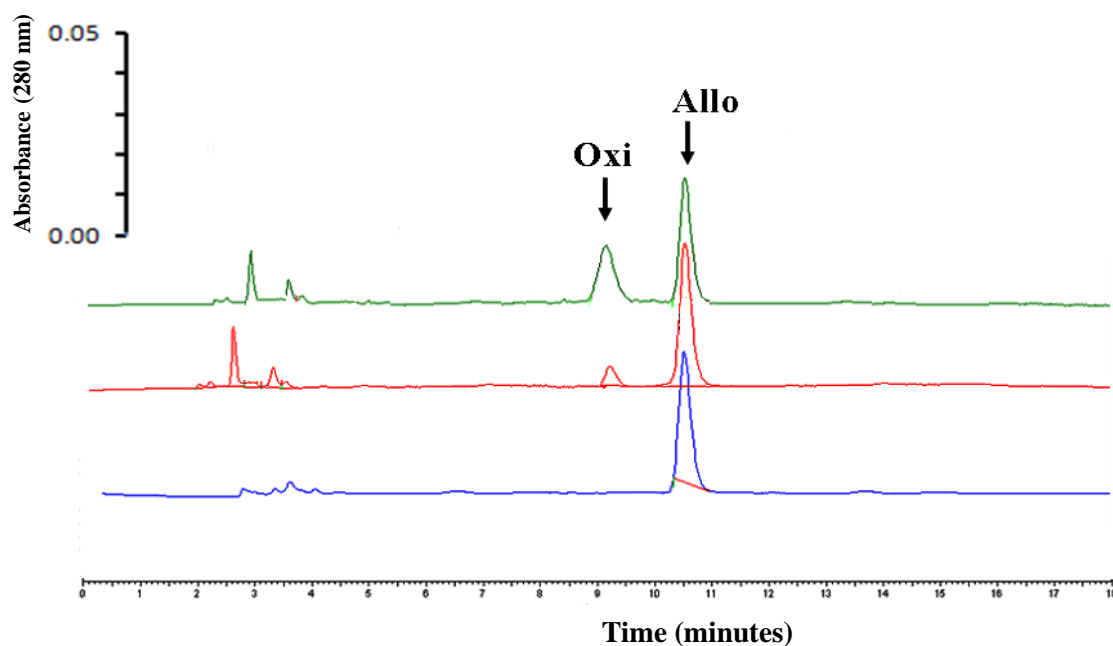


Figure 40: HPLC analysis of the *in vitro* biotransformation of allopurinol by *ry*⁵⁰⁶ cytosol strain.

Blue line is a zero minute incubation. Red and green lines are analytes from 30 and 120 minutes incubations. HPLC chromatograms are offset on the vertical axis to allow comparison between the different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

The enzyme activity of the *in vitro* allopurinol oxidation in the different *Drosophila melanogaster* strains are presented in the following table.

Table 24: Allopurinol oxidase activity in cytosol of *Drosophila melanogaster* strains.

Enzyme activity in cytosolic <i>Drosophila</i> strains (nmol converted in 30 minutes per mg protein of <i>Drosophila</i> cytosol strains)				
Canton-S with NAD ⁺	Canton-S without NAD ⁺	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-fl</i>
10.5 ± 1.5	3.5 ± 0.4	3.6 ± 0.6	N.D.	N.D.

Data represents mean ± S.D. of three independent experiments. N.D.: not detectable.

3.2.3. HPLC analysis of *D. melanogaster* strains with non purine and N-heterocyclic compounds.

The substrates used to evaluate AO activity in this research included the exogenous (drugs): cinchonine (anti-malaria drug), methotrexate (anti-cancer drug) and pyrazinamide (tuberculosis drug). *In vitro* incubations with Canton-S, *ry*⁵⁰⁶ and *ma-l* strains were carried out at several incubation times using cytosol with 0.1 mM final substrate concentration.

The pyrazinamide was incubated with Canton-S with and without NAD⁺ and HPLC analyses were carried out. After incubation with Canton-S cytosol with or without NAD⁺ the HPLC chromatogram showed a small putative 5-hydroxypyrazinamide peak (Figure 41 a and b). This indicated that it was a substrate using both conditions tested. Because of the 5-hydroxypyrazinamide is not commercially available, it was not possible to identify it due to the low level of 5-hydroxypyrazinamide in complex biological matrix. No results were detected by LC-MS. The chromatogram (Figure 42) showed that the XOR-null *ry*⁵⁰⁶ strain can also convert pyrazinamide to 5-hydroxypyrazinamide with O₂ as an electron acceptor indicating AO involvement in its conversion.

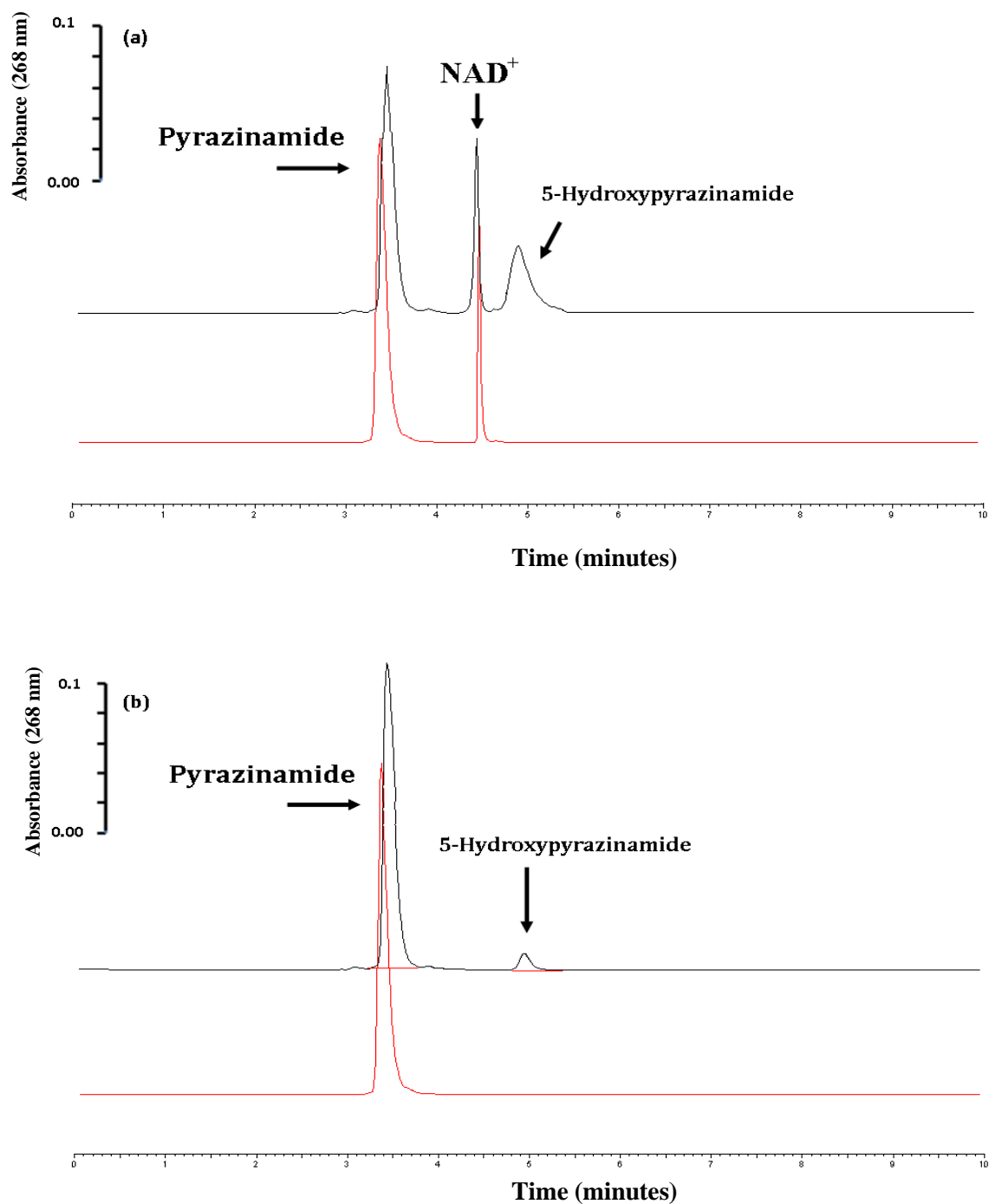


Figure 41: HPLC analysis of the *in vitro* biotransformation of pyrazinamide by Canton-S cytosol strain at 37°C with and without NAD^+ .

(a) Incubation with NAD^+ (b) Incubation without NAD^+ . Red and black are representative chromatograms produced at a zero and 240 minutes respectively. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μm) and eluted with a 20 mM KH_2PO_4 : acetonitrile (95: 5) as mobile phase. The wavelength of the detection was 268 nm.

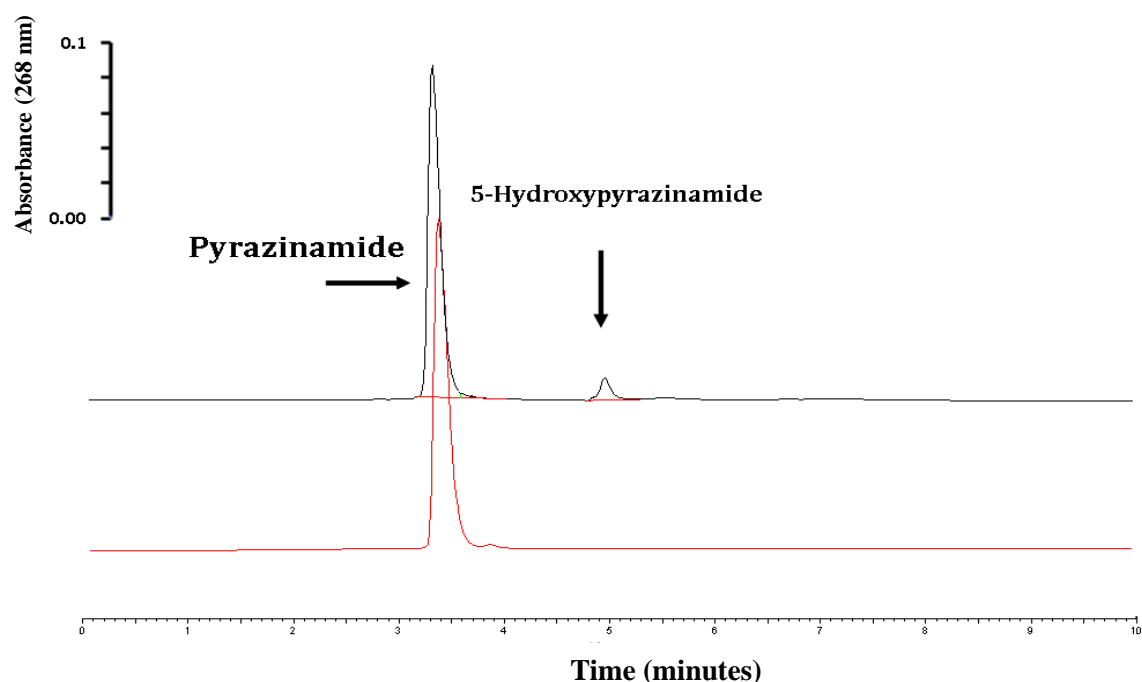


Figure 42: HPLC analysis of the *in vitro* biotransformation of pyrazinamide by *ry*⁵⁰⁶ *Drosophila melanogaster* cytosol at 37°C.

Blue line is a HPLC chromatogram of a zero minute incubation. Black line is a HPLC chromatogram at 240 minutes incubation. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 µm) and eluted with a 20 mM KH₂PO₄: acetonitrile (95: 5) as mobile phase. The wavelength of the detection was 268 nm.

When the N-heterocycle cinchonine was incubated with *Drosophila* cytosol from Canton-S and rosy strains one metabolite of cinchonine was detected by HPLC (Figure 43). Because its metabolite 2-cinchoninone is not commercially available; LC-MS was used to check the identity of the cinchonine metabolite. Unfortunately due to the low level of 2-cinchoninone in complex biological matrix no result was obtained to confirm its identity.

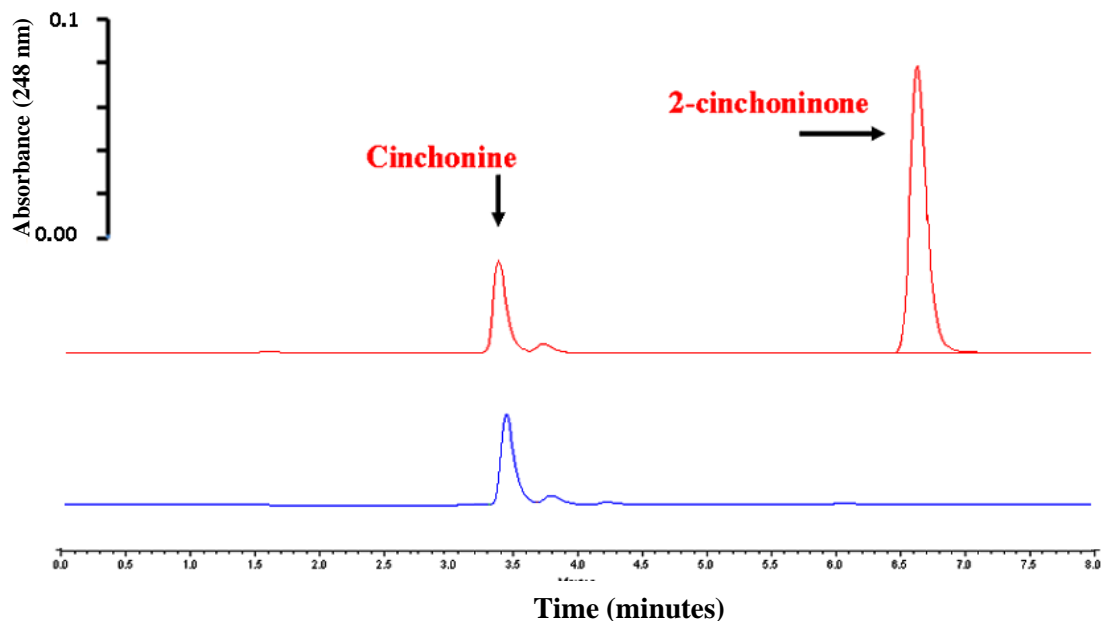


Figure 43: HPLC analysis of the *in vitro* biotransformation of cinchonine to 2'-cinchoninone by rosy cytosol.

Blue line is a HPLC chromatogram of a zero minute incubation. Red line is a HPLC chromatogram at 120 minutes incubation. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.4% w/v $\text{CH}_3\text{COONH}_4$: acetonitrile (65: 35) as mobile phase. The wavelength of the detection was 248 nm.

When methotrexate was tested as a substrate to the *Drosophila* cytosol no detectable hydroxylation of methotrexate (MTX) was observed with *Drosophila* cytosol even with extended incubations of 8 hrs (Figure 44 a). In contrast control incubations with rabbit cytosol showed demonstrable activity by oxidation MTX to its metabolite (7-hydroxymethotrexate) (Figure 44 b). These results confirmed that *Drosophila* AO has no activity toward MTX as a substrate.

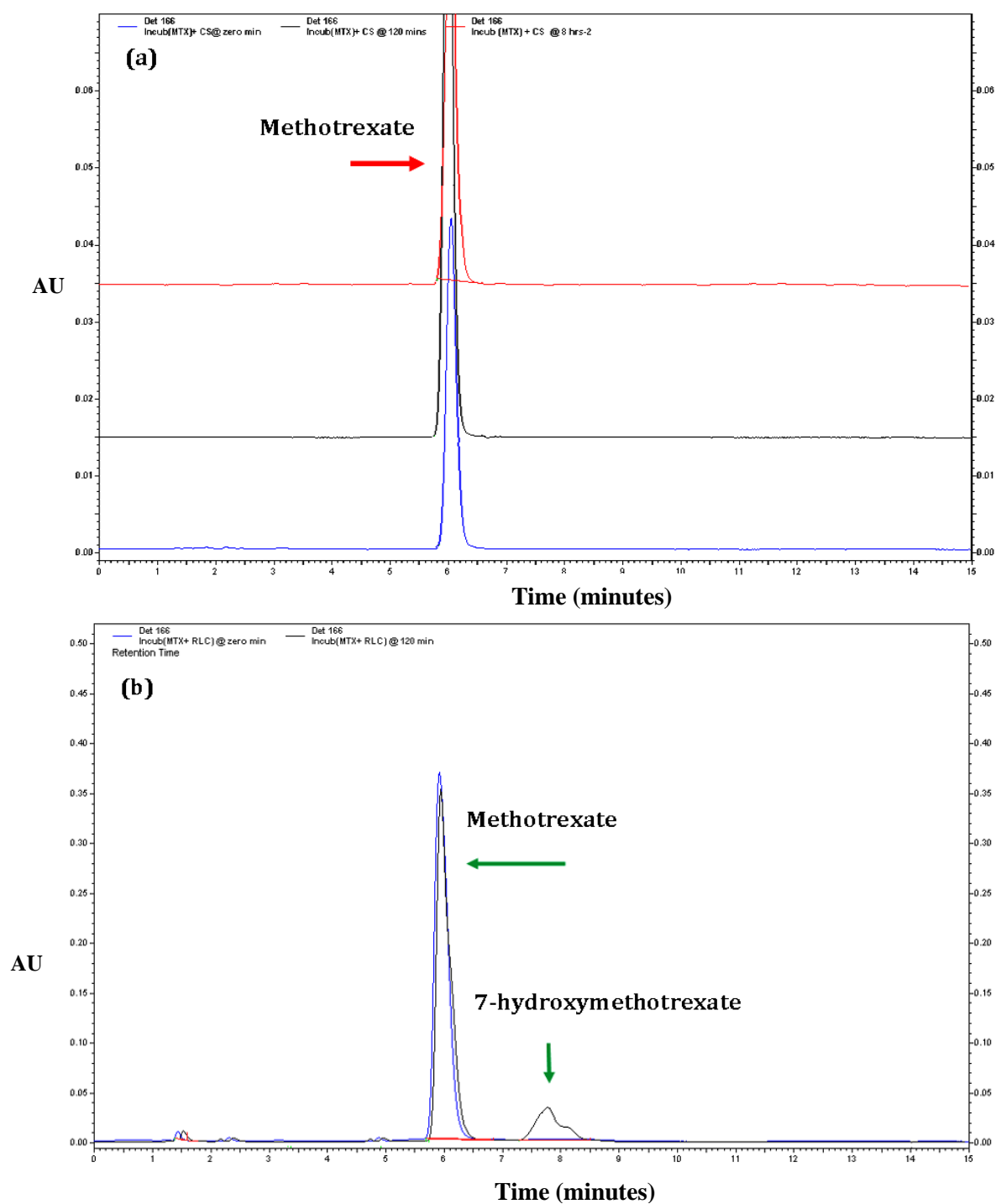


Figure 44: HPLC analysis of analytes produced following the *in vitro* incubation of methotrexate with *Drosophila* and rabbit liver cytosol.

(a) Canton-S cytosol. (b) Rabbit liver cytosol. Blue line is a HPLC chromatogram of a zero minute incubation. Black line is a HPLC chromatogram at 120 minutes and red line for 480 minutes incubation. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1 M $\text{CH}_3\text{COONH}_4$: acetonitrile (92: 8) as mobile phase. The wavelength of the detection was 305 nm.

When cinchonine, methotrexate and pyrazinamide were incubated with maroon-like (*mal-1* and *mal-f1*) cytosol even for extended incubation periods, HPLC analyses carried out did not detect any metabolites being formed.

The enzyme activity of the *in vitro* pyrazinamide and cinchonine oxidation in the different *Drosophila* strains is presented in table 25.

Table 25: Pyrazinamide and cinchonine oxidase activity in cytosol of *Drosophila melanogaster* strains.

AO and XOR substrate	Enzyme activity in cytosolic <i>Drosophila</i> strains (nmol converted in 120 minutes per mg protein of <i>Drosophila</i> cytosol strains)				
	Canton-S without NAD ⁺	Canton-S with NAD ⁺	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-f1</i>
Pyrazinamide	16.8 ± 5.0	36.0 ± 11.0	34.8 ± 10.6	N.D.	N.D.
Cinchonine	27.6 ± 10.2	-	24.0 ± 10.8	N.D.	N.D.

Data represents mean ± S.D. of three independent experiments. -: not tested. N.D.: not detectable.

3.2.4. HPLC *in vitro* analysis of *D. melanogaster* strains with endogenous vitamins and vitamin metabolites.

The endogenous vitamins B6 (pyridoxal), A (*all-trans* retinal) and nicotinamide B3 metabolite (N¹-methylnicotinamide) were also tested as a substrate of the different *Drosophila* strains. Pyridoxic acid and retinoic acid were formed by the action of AO in Canton-S and *ry*⁵⁰⁶ strains. Canton-S and *ry*⁵⁰⁶ strains had similar activity towards these compounds whereas *ma-l* strains had no detectable activity (Figure 45 and Table 26).

Two N1-methylnicotinamide products (Figure 46) were observed that were probably N¹-methyl-2-pyridone-5-carboxamide (2-PY) and N¹-methyl-4-pyridone-3-carboxamide (4-PY) as previously observed with rat and mice (Kitamura *et al.*, 2008; Shibata *et al.*, 1988; Stanulovic & Chaykin, 1971; Sugihara *et al.*, 2006).

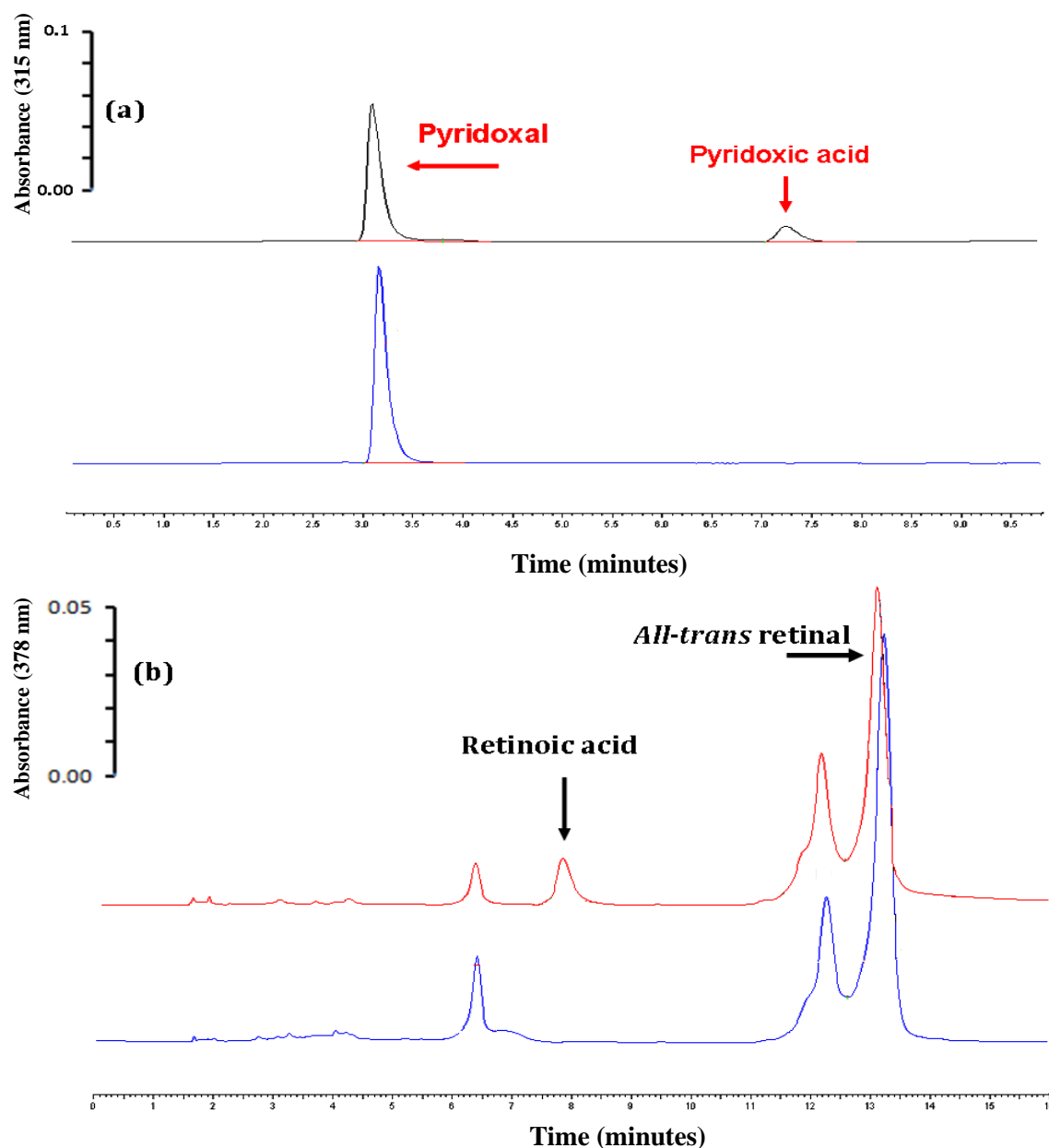


Figure 45: HPLC analysis of the *in vitro* biotransformation of endogenous compounds (pyridoxal and *all-trans* retinal) by Canton-S cytosol.

HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. (a) Incubation Canton-S cytosol with pyridoxal at 37 °C for 0 (blue line) and 60 minutes (red line). Analytes were injected onto a C-18 column (Kromasil 5 μ m) using methanol: 60 mM Na_2HPO_4 (25: 75) as mobile phase. The wavelength of the detection was 315 nm. (b) Incubation Canton-S cytosol with *all-trans* retinal. Blue line is a zero minute incubation and red line for 60 minutes incubation. Analytes were injected onto a C-18 column (LiChrosphere 5 μ m) and eluted with a 30 mM $\text{CH}_3\text{COONH}_4$: acetonitrile (30: 70) as mobile phase. The wavelength of the detection (DAD) was 378 nm.

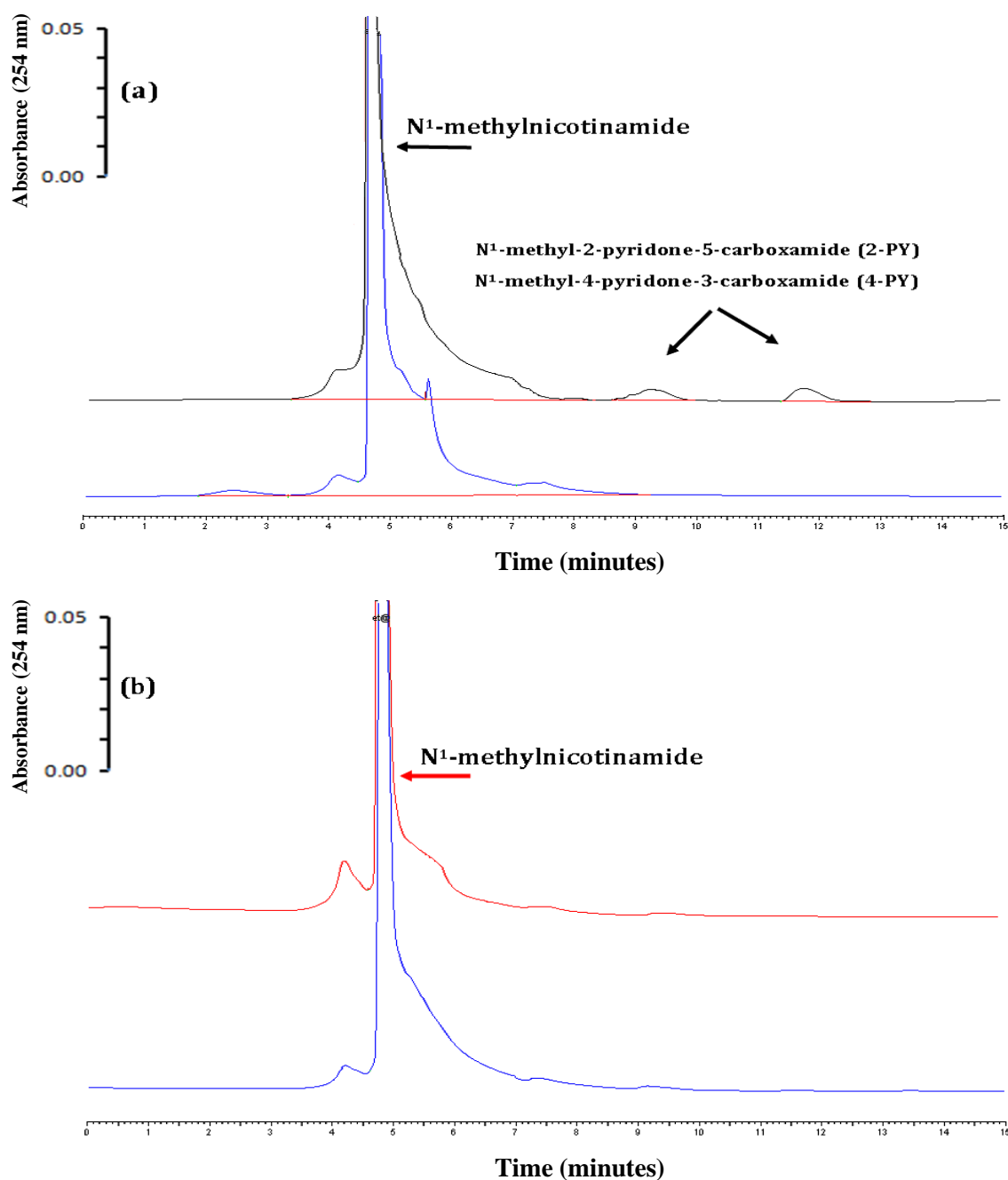


Figure 46: HPLC analysis of the *in vitro* biotransformation of N^1 -methylnicotinamide by Canton-S cytosol.

(a) HPLC chromatogram of the oxidation of N^1 -methylnicotinamide by Canton-S cytosol at a zero minute incubation (blue line) and 120 minutes incubation (black line). (b) HPLC chromatogram of the oxidation of N^1 -methylnicotinamide by maroon-like cytosol. Blue line is a HPLC chromatogram of a zero minute. Red line is a HPLC chromatogram of 120 minutes incubation. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analytes were injected onto a C-18 column (Spherisorb; CNRP 5 μ m) and eluted with a 0.8 % v/v formic acid: acetonitrile gradient as described in section 2.5.3 as mobile phase. The wavelength of the detection was 254 nm.

When pyridoxal, *all-trans* retinal and N¹-methylnicotinamide were incubated with maroon-like (*mal-1* and *mal-f1*) cytosol even for extended incubation periods, no metabolites were detected.

The enzyme activity of the *in vitro* endogenous vitamin oxidation in the different *D. melanogaster* strains is summarised in table 26.

Table 26: The oxidase activity towards pyridoxal, *all-trans* retinal and N¹-methylnicotinamide by cytosol of *Drosophila* strains.

AO substrate	Enzyme activity in cytosolic <i>Drosophila</i> strains (nmol converted in 10 minutes per mg protein of <i>Drosophila</i> cytosol strains)			
	Canton-S	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-f1</i>
Pyridoxal	17.5 ± 1.8	16.6 ± 1.7	N.D.	N.D.
<i>All-trans</i> retinal	7.1 ± 0.5	7.5 ± 0.6	N.D.	N.D.
N ¹ -methylnicotinamide	9.7 ± 1.9	9.9 ± 2.1	N.D.	N.D.

Data represents mean ± S.D. of three independent experiments. N.D.: not detectable.

3.3. Investigation of the chemoprotective role of molybdo-flavoenzymes against xenobiotic toxicity

The study of chemoprotective role of MFEs was performed by adding a variety of known AO and XOR substrates to *Drosophila* media. Percentage survival was assessed over a one to five weeks time course depending on the compounds being scrutinised (see methods sections for protocol used). In addition to testing the toxicity of compounds analytical tests were also performed to assess the ability of the different *Drosophila* strains to metabolise the compounds *in vivo* using the HPLC techniques developed for *in vitro* studies (section 2.5). All samples for HPLC assays were prepared and analysed by HPLC as described in section 2.5.4 with flies exposed to the potential toxicant for 4 days. In order to check the effect of the fly extract on the retention time of compounds, extracts were spiked with each of the analytes. In all cases no difference in retention time was found. Spiking was also carried out to confirm peak identity as well as using a diode array detector (DAD) when appropriate.

3.4.1. Endogenous purine content of *Drosophila melanogaster* strains.

Prior to assessing exogenous purine metabolism HPLC profiles were obtained for endogenous purine content of all *Drosophila* strains. This is shown in figure 47. As expected there was a demonstrable lack of uric acid and elevated xanthine in *ry*⁵⁰⁶ and *ma-l* strains compared with wild type and transgenic strains (Cote *et al.*, 1986; Finnerty *et al.*, 1979).

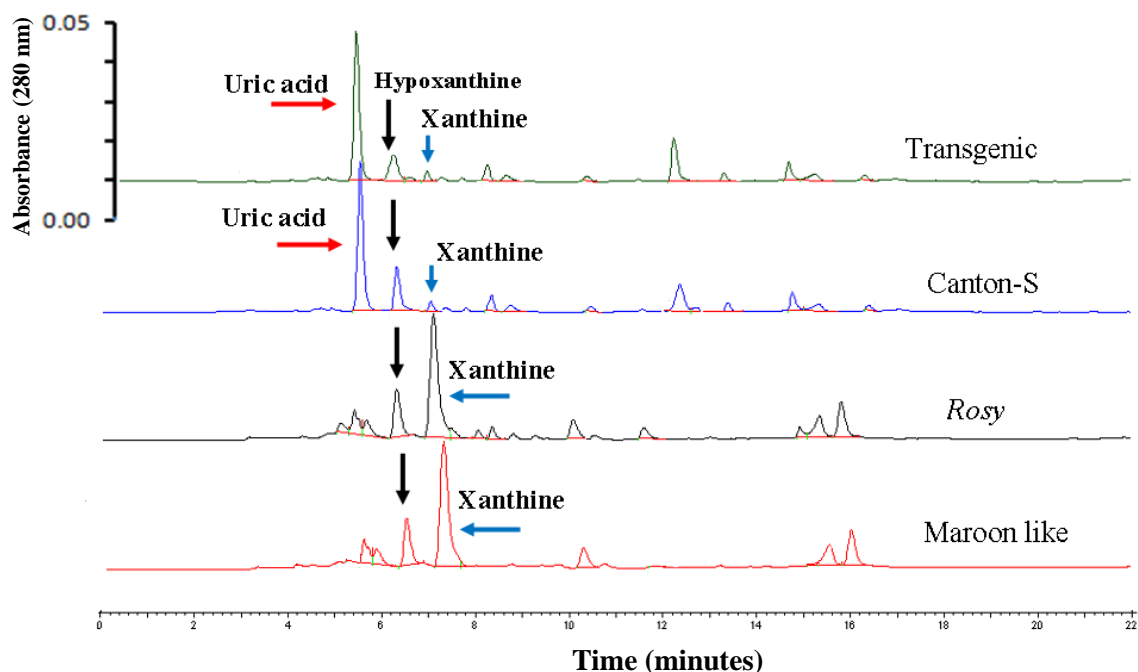


Figure 47: HPLC analysis of endogenous purines in untreated *Drosophila melanogaster* strains.

Canton-S (blue line), transgenic (green line), *ry*⁵⁰⁶ (black line) and maroon-like (red line). HPLC chromatograms are offset on the vertical axis to allow comparison between different *Drosophila* strains. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

Comparison of the purine levels of mutant *Drosophila* strains (rosy and maroon-like) (Table 27) with the normal *Drosophila* strain (Canton-S) indicated that the xanthine to hypoxanthine ratio was approximately 4 times the level of normal fly. The xanthine level of these mutants was 7 fold higher than in the normal strain, but no significant difference in the hypoxanthine level was seen. Rosy and maroon-like Mutant strains had no detectable uric acid.

Table 27: Xanthine, hypoxanthine and uric acid content of wild type, transgenic and mutant *D. melanogaster* strains.

	Strains			
	<i>ry</i> ⁵⁰⁶	Maroon-like	Canton-S	Transgenic
Xanthine (nmol/fly)	0.69 ± 0.11	0.71 ± 0.10	0.11 ± 0.007	0.10 ± 0.005
Hypoxanthine (nmol/fly)	0.19 ± 0.03	0.17 ± 0.02	0.16 ± 0.05	0.12 ± 0.03
Uric acid (nmol/fly)	N.D.	N.D.	0.82 ± 0.12	0.80 ± 0.10
Xanthine: hypoxanthine ratio	3.7	4.1	1.4	1.2

N.D not detected. Data represents mean ± S.D. of three independent experiments.

3.3.2. Investigation of the chemoprotective role of molybdo-flavoenzymes with purine and purine based drugs in *D. melanogaster* strains.

Following analysis of endogenous purines *Drosophila* were kept on either 20 mM or 100 mM caffeine (1, 3, 7-trimethylxanthine), theophylline (1, 3-dimethylxanthine), paraxanthine (1, 7-dimethylxanthine) and theobromine (3, 7-dimethylxanthine) for up to 2 weeks. The results of the toxicity study are presented in figure 48. All methylxanthines with the exception of paraxanthine tested were significantly more toxic to XOR-deficient *rosy* and *ma-l* strains than wild type (Canton-S) or transgenic (T1 and T2) strains at 20 mM see figure 48. As there was no toxic effect at 20 mM paraxanthine with any strain (data not shown) a 100 mM dose was used that showed a similar pattern of toxicity to the strains tested with XOR- deficient strains having a much more pronounced death rate.

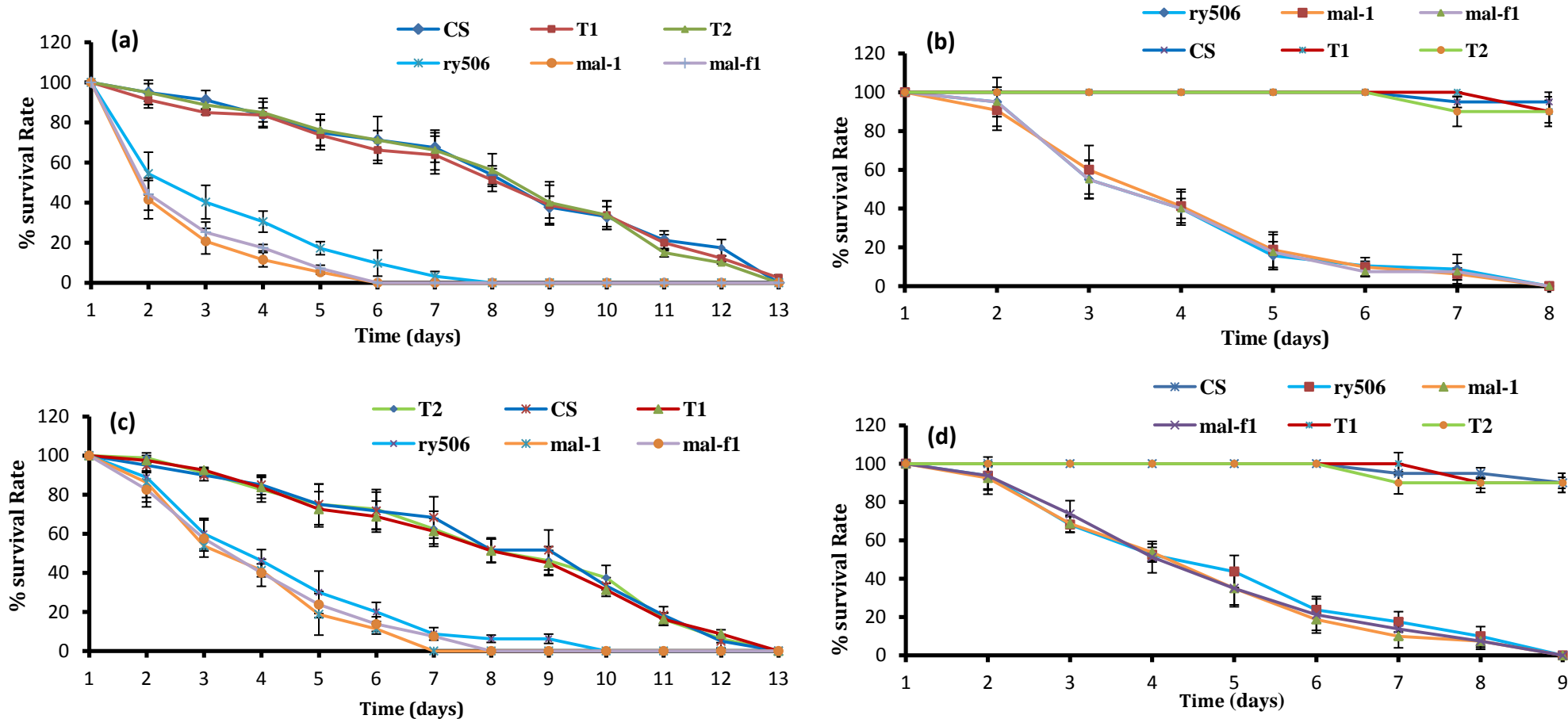


Figure 48: Toxicity of methyl-xanthines to *Drosophila melanogaster* strains (a) caffeine (20 mM), (b) theobromine (20mM), (c) theophylline (20 mM) and (d) paraxanthine (100 mM).

Each vial contained twenty, 0-3 days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

Analysis of metabolites obtained from all fly strains incubated with these methylxanthines indicated that apart from the methylated xanthine added to the media and the previously identified endogenous purines there were no peaks that represented any of the known eight caffeine metabolites used as standards. Peaks found at 12.6, 13.3 and 15.0 minute retention times were unidentified compounds that also appeared in untreated fly extracts. Canton-S HPLC profiles are shown for illustration figures 49 and 50.

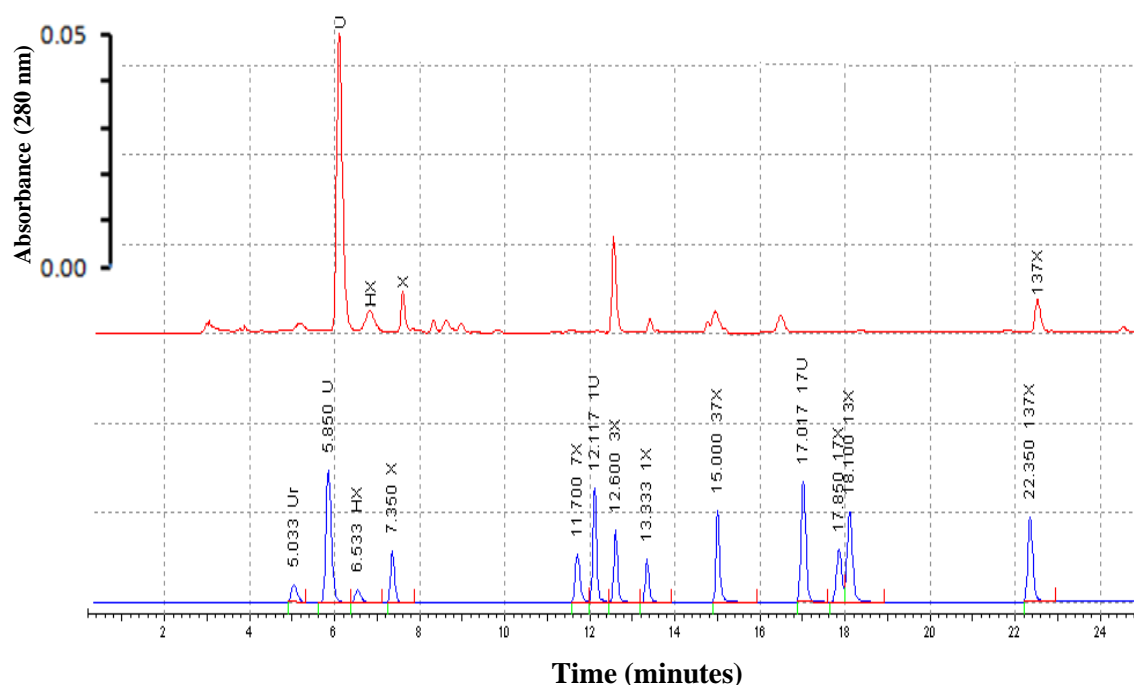


Figure 49: HPLC analysis wild type Canton-S *Drosophila melanogaster* extracts after 4 days treatment with caffeine.

HPLC chromatograms are offset on the vertical axis to allow comparison between wild type Canton-S and caffeine metabolite and endogenous purine standards. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm. HPLC chromatograms of extracts from Canton-S treated for 4 days with 20 mM caffeine in media (red line). 250 μ M caffeine metabolite and endogenous purine standards (blue line). **Abbreviations:** Ur = uracil; U = uric acid; HX = hypoxanthine; X = xanthine; 7X = 7-methylxanthine; 1U = 1-methyluric acid; 3-MX = 3-methylxanthine; 1X = 1-methylxanthine; 37X = 3,7-dimethylxanthine or theobromine; 17U = 1,7-dimethyluric acid; 17X = 1,7 dimethylxanthine or paraxanthine; 13X = 1,3-dimethylxanthine or theophylline and 137X = 1,3,7- trimethylxanthine or caffeine.

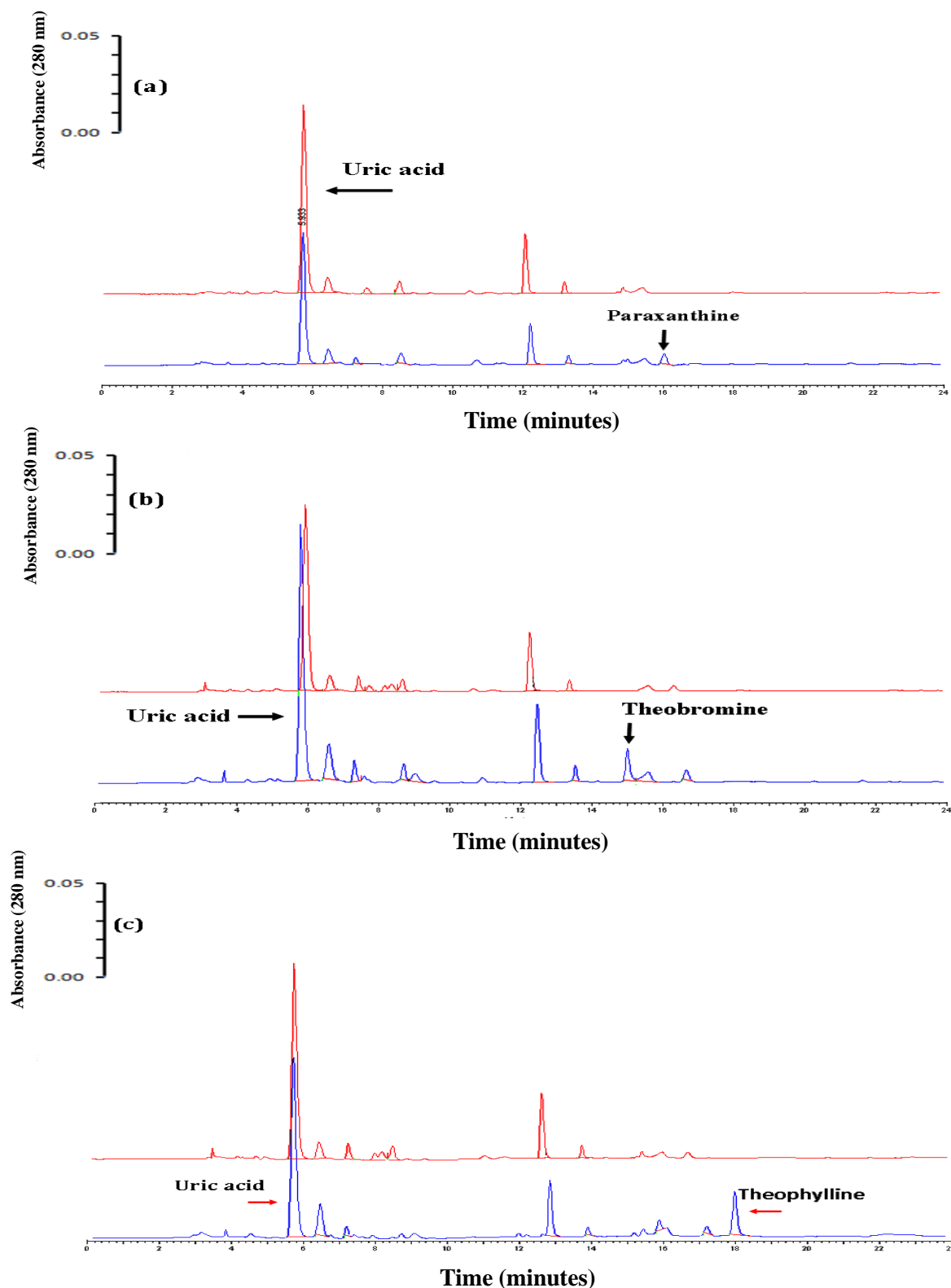


Figure 50: HPLC chromatogram of purines found in Canton-S *Drosophila* after 4 days of treatment with paraxanthine, theobromine and theophylline.

Red line is untreated Canton-S *Drosophila* and blue line is Canton-S treated with methylxanthines. (a) paraxanthine, (b) theobromine and (c) theophylline. HPLC chromatograms are offset on the vertical axis to allow comparison between the treated and untreated flies. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm.

As well as the drugs theophylline and caffeine the toxicity of several other purine or purine analog drug substrates were assessed. On comparing the percentage survival of Canton-S and transgenic with *ry*⁵⁰⁶, *mal-1* and *mal-f1* strains treated with the anti-leukemic drug 6-mercaptopurine the percentage survival of wild type and transgenic was slightly greater than *ry*⁵⁰⁶ and maroon-like strains (Figure 51). From statistical tests comparing the Canton-S (wild type), transgenic and mutant strains (*ry*⁵⁰⁶, *mal-1* and *mal-f1*) found a highly significant difference ($P < 0.05$) between XDH active (Canton-S and transgenic) and XDH inactive mutant strains (*ry*⁵⁰⁶, *mal-1* and *mal-f1*) (Appendix 4; Tables 3). HPLC of 6-mercaptopurine treated flies of all strains revealed no detectable metabolites after 4 days (data not shown).

As well as 6-mercaptopurine the purine analog anti-gout drug allopurinol was tested. The percentage survival for all strains exposed to allopurinol containing media can be seen in figure 51. It was observed that the strains harbouring mutations in XOR or MCS genes had an increased sensitivity to the toxicity of allopurinol. 20 mM allopurinol was most toxic to maroon-like strains with the effect being much less pronounced with the *ry*⁵⁰⁶ strain. As there was a less pronounced effect on the rosy strain with 20 mM allopurinol, in order to investigate if allopurinol had a more pronounced effect at higher concentration 100 mM allopurinol was tested. This demonstrated that allopurinol at this elevated concentration led to all *ry*⁵⁰⁶ flies dying by day 15 whereas XOR-active strains (Canton-S and transgenic) had 100% survivorship at this time point.

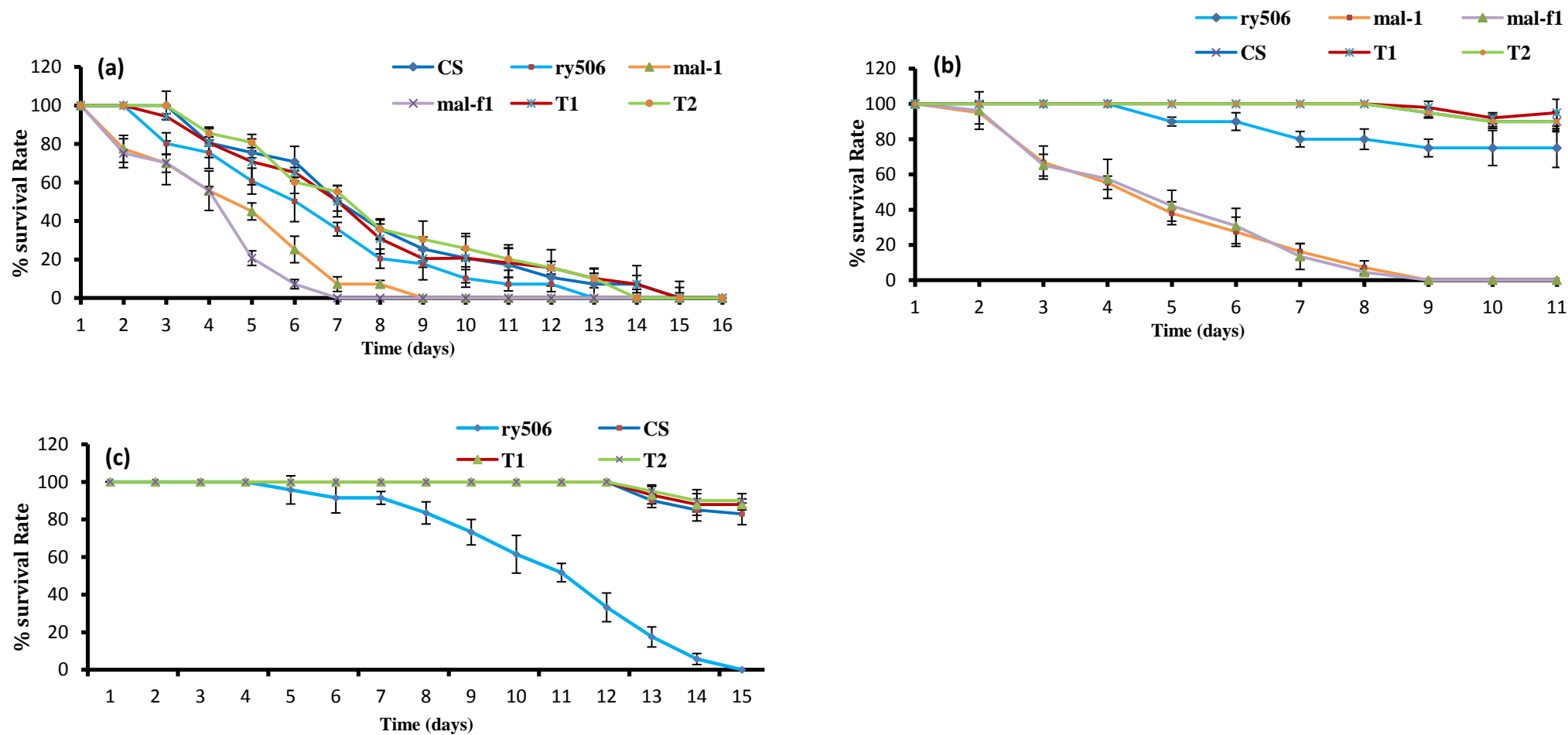


Figure 51: Toxicity of 6-mercaptopurine and allopurinol to wild type, transgenic and mutant of *Drosophila melanogaster* strains.

(a) 6-mercaptopurine (20mM). (b) Allopurinol (20mM). (c) Allopurinol (100 mM). Each vial contained twenty, 0-3days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

HPLC analysis of fly extracts demonstrated that Canton-S, transgenic and rosy strain generated oxipurinol (Figure 52) in 20 mM allopurinol media whereas the *ma-l* strain had no detectable metabolite.

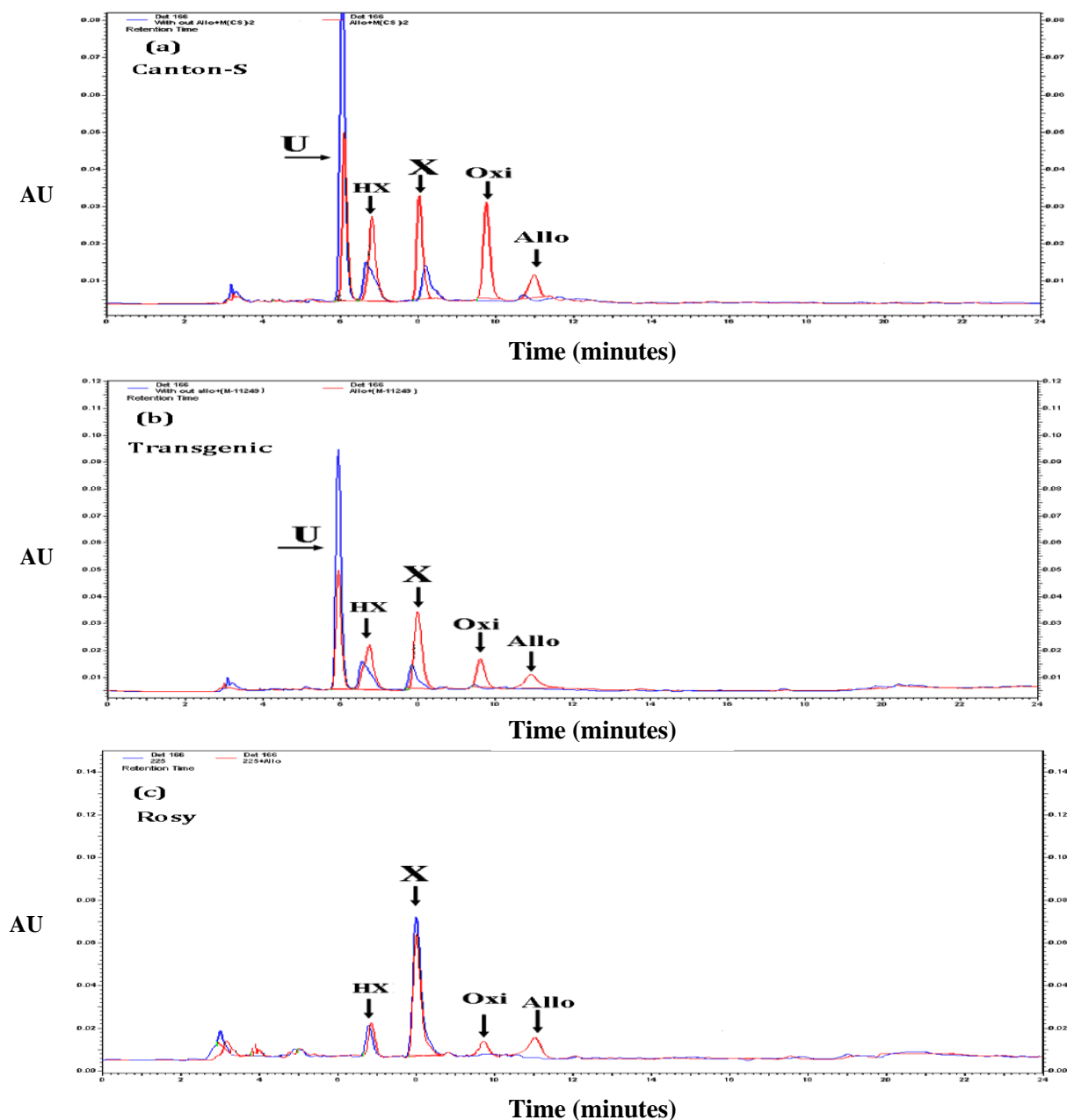


Figure 52: HPLC analysis of allopurinol metabolism in *Drosophila melanogaster* strains.

The red line chromatogram is an extract from allopurinol treated *Drosophila* strains. The blue line chromatogram shows the sample of untreated *Drosophila* strains. (a) Canton-S, (b) transgenic and (c) rosy. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.1% v/v glacial acetic acid: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 280 nm. **Abbreviations:** U = uric acid; HX = hypoxanthine; X = xanthine; Oxi = oxipurinol and Allo = allopurinol.

The quantification of allopurinol and oxipurinol in each of the strains is shown in table 28.

Table 28: Quantification of allopurinol and oxipurinol in fly extracts after 4 days of 20 mM allopurinol treatment.

Strain	nmol/fly		% Metabolite (Oxipurinol)
	Allopurinol	Oxipurinol	
Canton-S <i>ry</i> ⁵⁰⁶ <i>mal-1</i>	0.28 ± 0.04	0.70 ± 0.12	71.4 ± 10.5
	0.30 ± 0.05	0.41 ± 0.07	57.7 ± 8.6
	0.91 ± 0.13	N.D	-

Data represents mean ± S.D. of three independent experiments. N.D.: not detectable.

It was also observed that allopurinol caused approximately 50 % decrease in uric acid and significant increase in xanthine and hypoxanthine in untreated Canton-S (Figure 52).

3.3.3. Investigation of the chemoprotective role of molybdo-flavoenzymes with toxicity of non-purine based N-heterocyclic compounds in *D. melanogaster* strains.

Several other non-purine based N-heterocyclic compounds were used in the toxicity studies. These included phenanthridine (an AO substrate and environmental pollutant), pyrazinamide (an AO and XOR substrate and anti-tuberculosis drug) and cinchonine (an AO substrate and anti-malarial). When these compounds were administered in fly media as described in section 2.4 the following graphs were obtained (Figure 53). This illustrates the percentage survival of *Drosophila* strains after treatment with 20 mM concentration of these compounds in media. The maroon-like strains appeared weaker as they had a higher mortality rate than that of the rosy, transgenic and wild type strains with all compounds. With phenanthridine and cinchonine there was no significant difference between *ry*⁵⁰⁶, transgenic and wild type strains (Appendix 4; Tables 3 and Figure 53). In contrast, pyrazinamide caused a faster death rate and highly significant difference ($P < 0.05$) when the *ry*⁵⁰⁶ strain was

compared to the two MFE-active strains and there was slightly significant difference than *ma-l* strains (Appendix 4; Tables 3 and Figure 53).

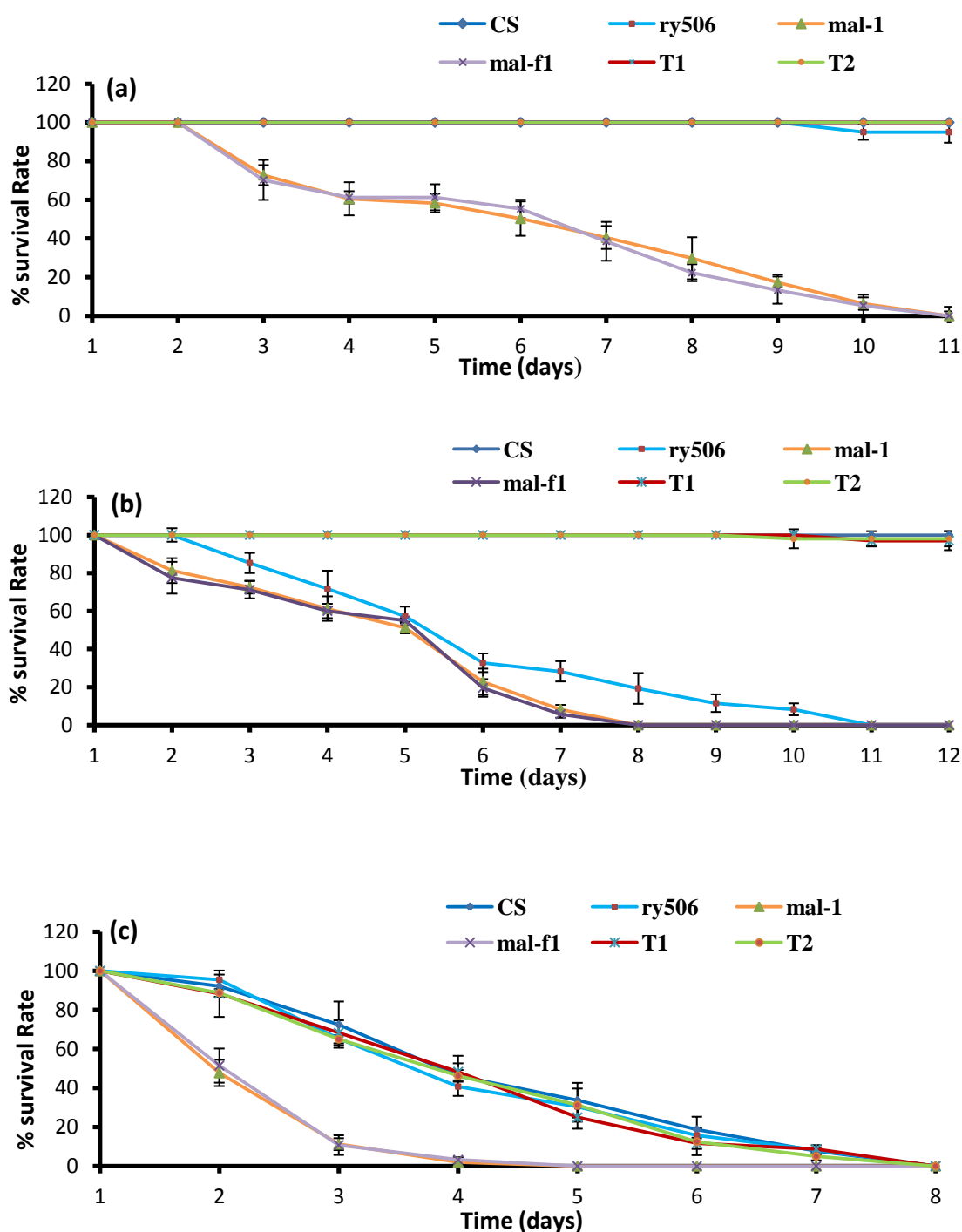


Figure 53: Toxicity of 20 mM phenanthridine, pyrazinamide and cinchonine to wild type, transgenic and mutant of *Drosophila melanogaster* strains.

(a) phenanthridine, (b) pyrazinamide and (c) cinchonine. Each vial contained twenty, 0-3days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

To determine the metabolic basis of the toxicities found the oxidation of phenanthridine, pyrazinamide and cinchonine with the different *Drosophila melanogaster* strains was examined in fly extracts. Both wild type (Canton-S) and rosy mutant (*ry*⁵⁰⁶) strains had significant quantities of 6(5H)-phenanthridone metabolite generated at day 4 of the experiment in contrast to the maroon-like strains that had no detectable metabolite (Figure 54 and Table 29). The quantification of phenanthridine and 6(5H)-phenanthridone in each strain is shown in table 29.

Table 29: Quantification of phenanthridine and 6(5H)-phenanthridone in fly extract after 4 days of 20 mM phenanthridine treatment.

Strain	nmol/fly		% metabolite (6(5H)-phenanthridone)
	Phenanthridine	6(5H)-phenanthridone	
Canton-S	0.21 ± 0.04	0.19 ± 0.03	47.5 ± 6.0
<i>ry</i> ⁵⁰⁶	0.25 ± 0.03	0.21 ± 0.04	46.5 ± 5.8
<i>mal-1</i>	0.75 ± 0.10	N.D.	-

Data represents mean ± S.D. of three independent experiments. N.D.: not detectable.

Pyrazinamide, a drug used to treat tuberculosis is metabolised to 5-hydroxypyrazinamide by the action of XOR and AO in mammals (Kitamura *et al.*, 2006; Moriwaki *et al.*, 1997). All strains under pyrazinamide treatment showed a similar profile in that there were no clear peaks of 5-hydroxypyrazinamide metabolite with any strains (data not shown). All strains of *Drosophila melanogaster* treated with cinchonine that is a known mammalian AO substrate were also analysed for metabolites. Canton-S and *ry*⁵⁰⁶ strains showed a metabolite peak at a retention time of 6.7 minutes in the chromatograms (see figure 54 as example of Canton-S strain). In theory this was 2-cinchoninone however due to the lack of commercial availability of this compound and the complexity of the biological sample that made the LC-MS result inconclusive it was therefore not possible to unequivocally identify the

metabolite found in this case. In contrast to Canton-S and *ry*⁵⁰⁶ strains there was no conversion of cinchonine to the putative 2-cinchoninone peak in maroon-like strains.

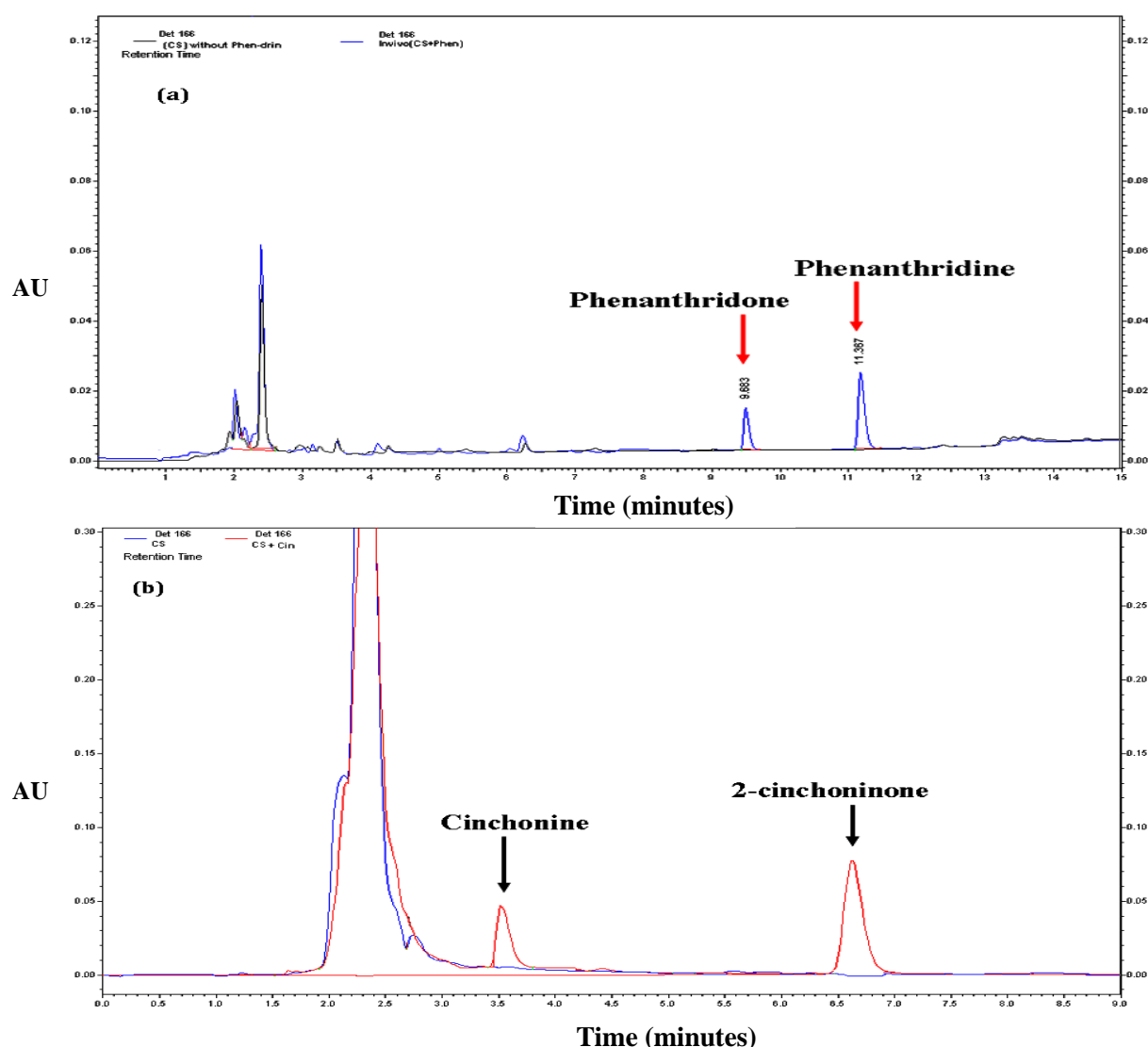


Figure 54: HPLC analysis of phenanthridine and cinchonine biotransformation in Canton-S *Drosophila melanogaster* strain.

(a) Phenanthridine biotransformation in Canton-S strain. The blue line is a chromatogram of the extract from phenanthridine treated Canton-S animals and the black line is a chromatogram of untreated Canton-S. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with water: acetonitrile gradient system as described in section 2.5.3 as mobile phase. The wavelength of the detection was 254 nm. (b) Cinchonine biotransformation in Canton-S strain. The red line is a chromatogram of the extract from cinchonine treated Canton-S animals and the black line is a chromatogram of untreated Canton-S. HPLC chromatograms are offset on the vertical axis to allow comparison between different incubation times. Analyte injected onto a C-18 column (Kromasil 5 μ m) eluted with a 0.4% w/v $\text{CH}_3\text{COONH}_4$: acetonitrile (65: 35) as mobile phase. The wavelength of the detection was 248 nm.

3.3.4. Investigation of the chemoprotective role of molybdo-flavoenzymes with vanillin and N¹-methylnicotinamide toxicity in *D. melanogaster* strains.

The results from the N¹-methylnicotinamide toxicity tests are shown in figure 55. Figure 55 demonstrates that the strains of *Drosophila* lacking aldehyde oxidase are less able to survive in media containing N¹-methylnicotinamide. When exposed to the N¹-methylnicotinamide in the media, maroon-like strains had a highly significant decreased survivorship compared to all other strains tested (Appendix 4; Tables 3). At day 9 all maroon-like flies were dead, whereas there was 100% survival of all other strains at this time point.

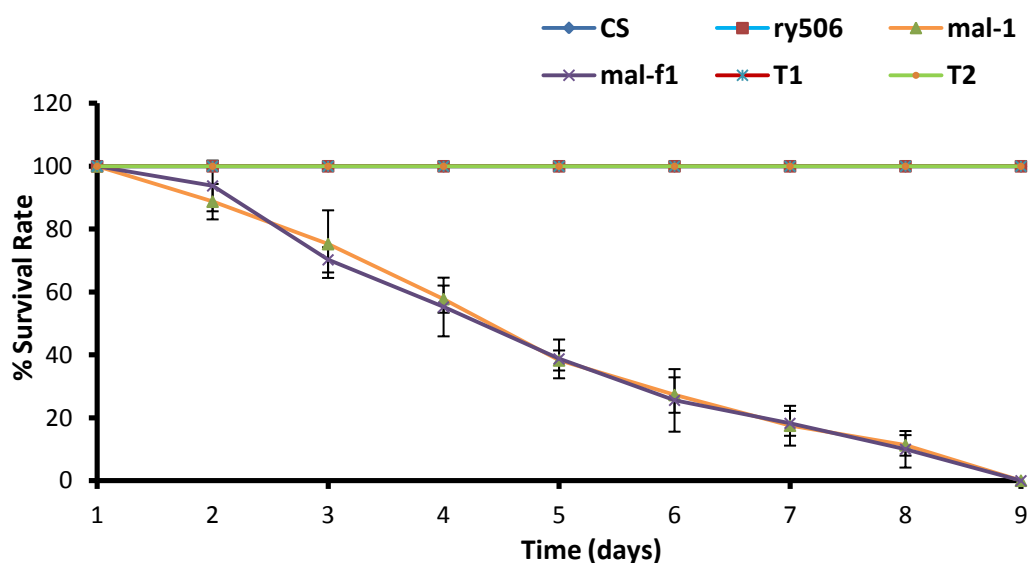


Figure 55: Toxicity of 20 mM N¹-methylnicotinamide to wild type, transgenic and mutant of *Drosophila melanogaster* strains.

Each vial contained twenty, 0-3days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

In order to test for the MFE-mediated metabolism of N¹-methylnicotinamide HPLC analysis was performed. Strains of *Drosophila melanogaster* treated with N¹-

methylnicotinamide were analysed by HPLC. Quantification of N¹-methylnicotinamide and its metabolites in all *Drosophila* strains treated with this compound revealed no detectable pyridine metabolites were detectable using the HPLC protocol described in section 2.5.3 (data not shown).

To determine if vanillin was toxic to *Drosophila* flies from each strain were exposed to vanillin in low and high concentrations (20 mM and 100 mM). The results of survivorship of the different *Drosophila* strains when exposed to the 20 mM of vanillin demonstrated that it was not toxic to any strains at this concentration when monitored for up to three weeks (Figure 56) compared to the normal lifespan of all *Drosophila* strains (see figure 56).

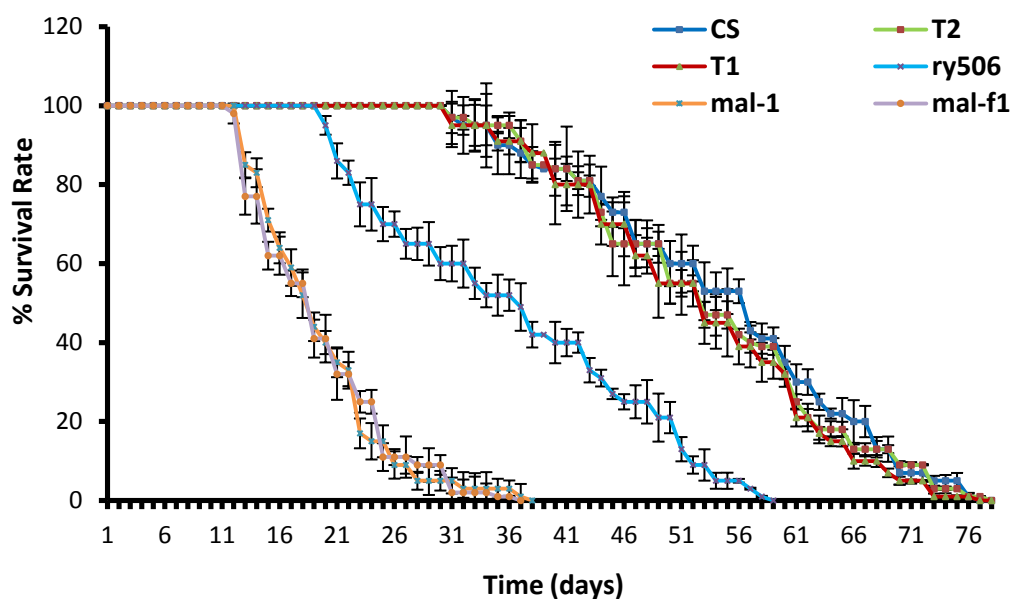


Figure 56: Toxicity of 20 mM vanillin to wild type, transgenic and mutant of *Drosophila melanogaster* strains.

Each vial contained twenty, 0-3 days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

Interestingly, the average lifespan of all strains treated with 20 mM vanillin concentration appeared to be slightly increased compared with to their normal mean

lifespan (Figures 56 and 57). However, according to the statistical analysis (Table 30), it was observed that the mean lifespan of all strains treated with 20 mM vanillin were no significant difference ($P > 0.05$).

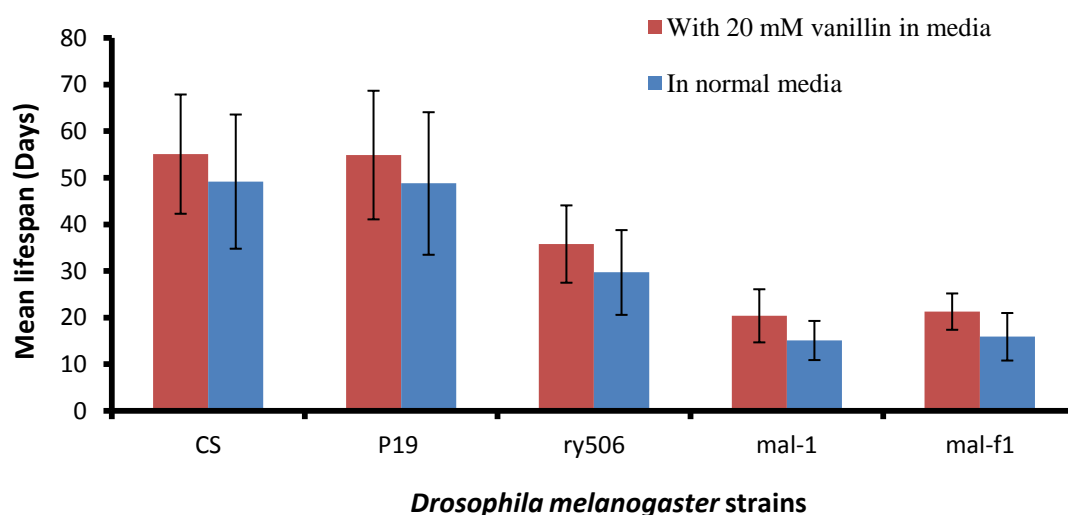


Figure 57: Comparison of the effect of 20 mM vanillin on lifespan of *Drosophila melanogaster* strains.

Data represents mean \pm S.D. of four sets of data. Data from table 32 for untreated flies (control) and figure 56.

Table 30: Statistical difference of mean lifespan between 20 mM vanillin treated and untreated *Drosophila* strains.

χ^2 value of <i>Drosophila melanogaster</i> lifespan				
Canton-S normal media	T1 normal media	<i>ry</i> ⁵⁰⁶ normal media	<i>mal-1</i> normal media	<i>mal-f1</i> normal media
Canton-S vanillin media	T1 vanillin media	<i>ry</i> ⁵⁰⁶ vanillin media	<i>mal-1</i> vanillin media	<i>mal-f1</i> vanillin media
0.60	0.46	0.96	0.86	1.0

(* $P < 0.05$ by 05 by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

The effects of vanillin observed at 100 mM was that the strains harbouring mutations of XOR and AO (*mal-1* and *mal-f1*) had a highly significant decreased survivorship when compared to the wild type, transgenic and *ry*⁵⁰⁶ strains (Figure 58 and Appendix 4; Table 3).

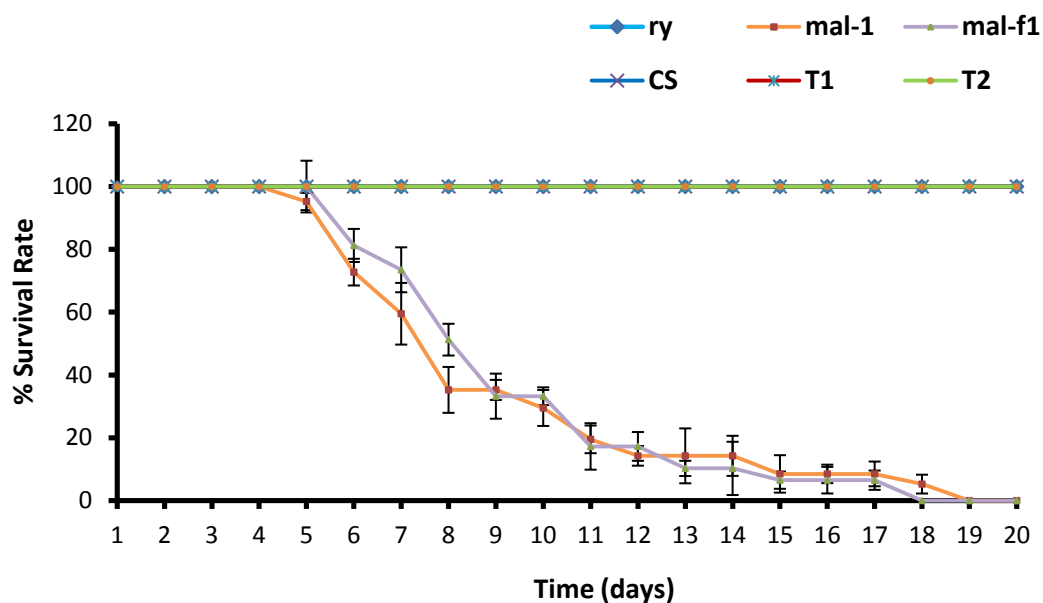


Figure 58: Toxicity of 100 mM vanillin to wild type, transgenic and mutant *Drosophila melanogaster* strains.

Each vial contained twenty, 0-3 days old male flies, each point represents mean \pm SD of four sets of data. CS = Canton-S. T1 and T2 = transgenic.

In order to test for the MFE-mediated metabolism of vanillin HPLC analysis was performed. Canton-S, transgenic and *ry*⁵⁰⁶ strains under vanillin treatment showed its metabolite vanillic acid (Figure 59). In contrast maroon-like strains displayed no detectable vanillic acid peak.

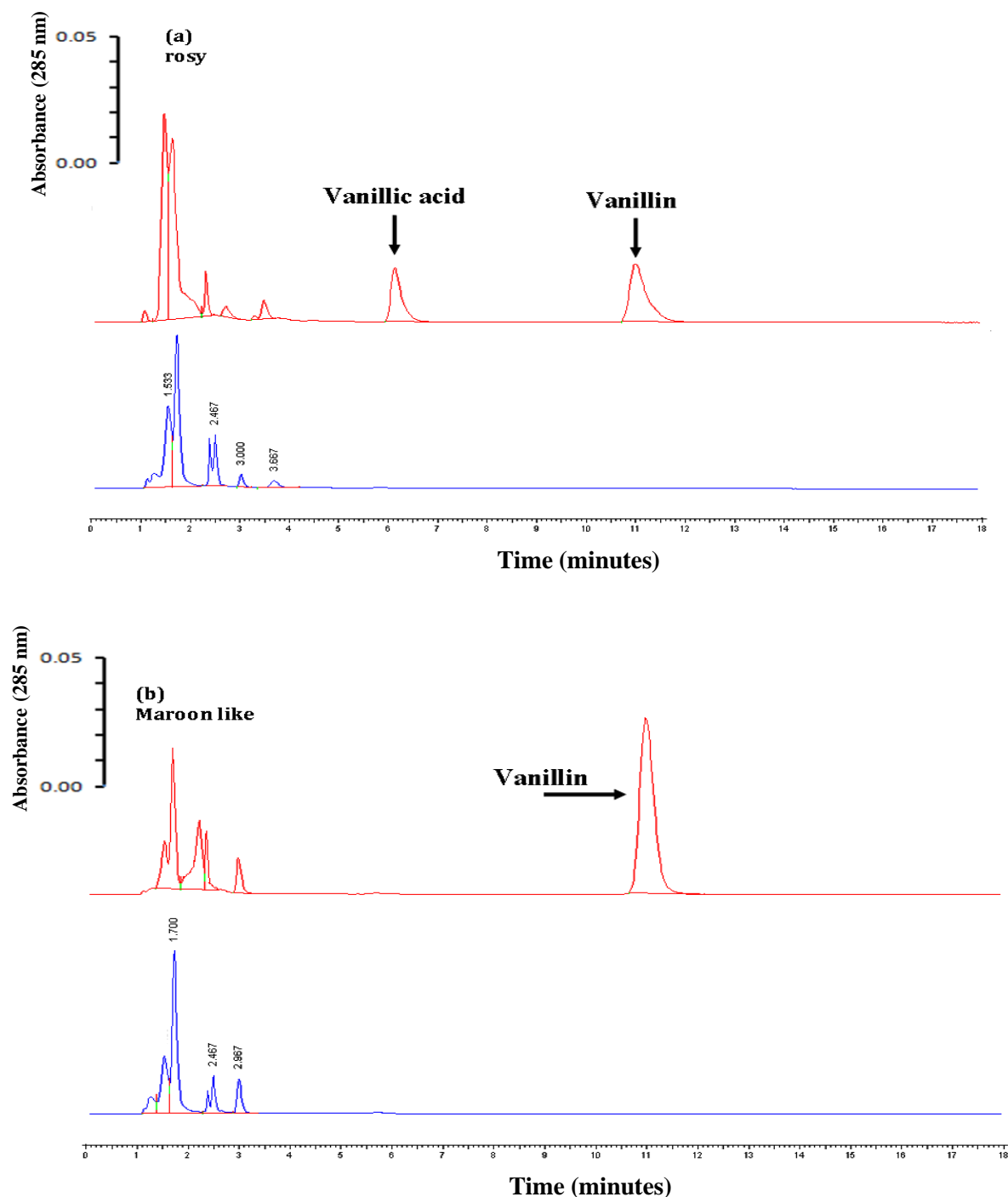


Figure 59: HPLC analysis of vanillin biotransformation in *Drosophila melanogaster* strains. (a) HPLC chromatogram showing the difference between *ry*⁵⁰⁶ treated (red line) and untreated with vanillin (blue line). (b) HPLC chromatogram of an extract from *mal-1* *Drosophila* strain treated with vanillin. The blue line chromatogram shows the sample of untreated *mal-1* *Drosophila*. The red line is the treated sample. HPLC chromatograms are offset on the vertical axis to allow comparison between different *Drosophila* strains. Analytes were injected onto a C-18 column (Kromasil 5 μ m) and eluted with a 0.22 M KH_2PO_4 : acetonitrile system (85: 15) as mobile phase. The wavelength of the detection was 285 nm.

The quantification of vanillin and vanillic acid in each of the strains is shown in table 31.

Table 31: Quantification of vanillin and vanillic acid in fly extract after 4 days of 20 mM vanillin treatment.

Strain	nmol/fly		% Metabolic (Vanillic acid)
	Vanillin	Vanillic acid	
Canton-S <i>ry⁵⁰⁶</i> <i>ma-l</i>	1.11 ± 0.20	2.37 ± 0.41	68.1 ± 11.1
	0.67 ± 0.10	3.56 ± 0.63	84.1 ± 9.3
	3.86 ± 0.72	N.D.	-

Data represents mean ± S.D. of three independent experiments. N.D: not detectable.

3.4. Lifespan Studies of molybdo-flavoenzymes-active and molybdo-flavoenzymes-deficient *Drosophila* strains.

The effect of MFE deficiencies on lifespan of MFE-active (Canton-S and transgenic) and MFE deficient (*ry*⁵⁰⁶ and *mal-l*) strains of *Drosophila* is shown in figure 60.

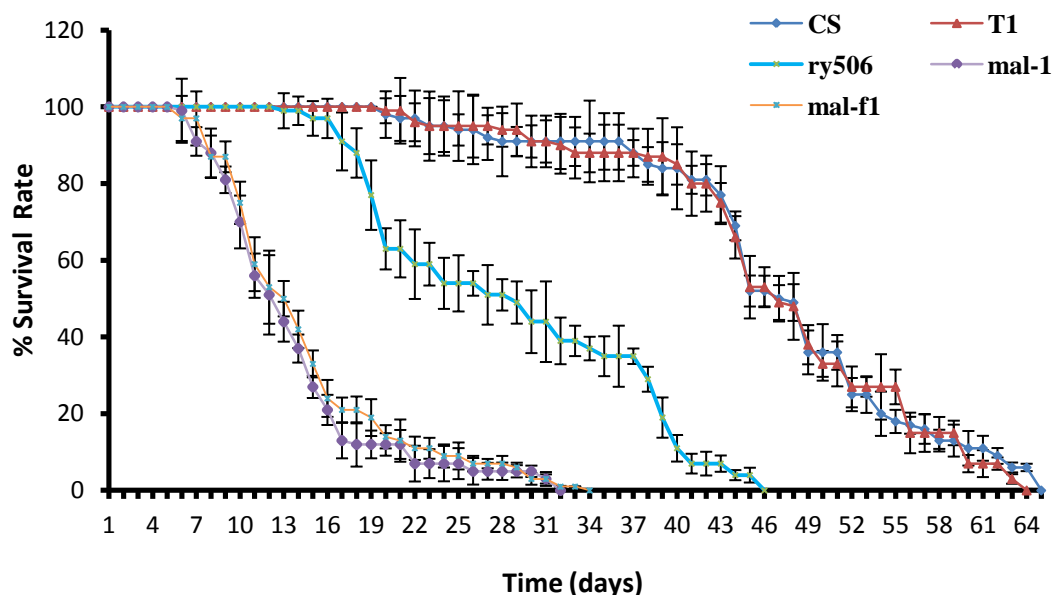


Figure 60: The lifespan of Canton-S, transgenic, *ry*⁵⁰⁶, *mal-l* and *mal-fl* strains.

Each vial = 20 male flies. Data represents mean \pm S.D five sets of data. CS = Canton-S. T1= transgenic.

The mean lifespan of all the strains tested are shown in table 28. According to the results, it was observed that the mean lifespan of the Canton-S, transgenic, *ry*⁵⁰⁶, *mal-l* and *mal-fl* strains were 49.2 ± 10.4 , 48.8 ± 9.3 , 29.7 ± 7.1 , 15.1 ± 3.2 and 15.1 ± 2.4 days \pm S.D respectively (Table 32). The graph shows the transgenic strains that have had the *ry* gene re-inserted (T1) have a lifespan which is similar to that of the Canton-S strain lifespan. The statistical analysis of the results showed that there were no difference in lifespan of Canton-S and transgenic strains with each test resulting in $P > 0.05$ values (Tables 32). The Canton-S strain survived for considerable longer than the

*ry*⁵⁰⁶ strain and maroon-like flies (*mal-1* and *mal-f1*). The *P* values for longevity of *ry*⁵⁰⁶ and the maroon-like strains are lower than (*P* < 0.05) value compared with Canton-S strain (Tables 32). In addition, the null XOR/AO strains (*mal-1* and *mal-f1*) had a reduced lifespan when compared with that the XOR null strain (*ry*⁵⁰⁶) strain. There was also a high level of significant difference between the rosy and maroon-like strains (Table 32).

Table 32: Mean lifespan and maximum life of wild type, transgenic and mutant strains.

Strain	N	Maximum Life of last fly (Days)	Mean lifespan Days \pm SD	χ^2 value of <i>Drosophila melanogaster</i> mean lifespan	
				Canton-S	<i>ry</i> ⁵⁰⁶
Canton-S	100	64	49.2 \pm 10.4	-	5.72*
T1	100	62	48.8 \pm 9.3	0.004	5.22*
<i>ry</i> ⁵⁰⁶	100	45	29.7 \pm 7.1	5.72*	-
<i>mal-1</i>	100	29	15.1 \pm 3.2	27.66*	18.41*
<i>mal-f1</i>	100	31	15.9 \pm 2.4	28.11*	18.75*

N: Total number of individuals. Data represents mean \pm S.D. of 5 sets of data. Statistical results of mean lifespan between *Drosophila* strains (**P* < 0.05 by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

From the results the effect of XOR and AO enzymes on longevity of *Drosophila* is clear. It can be seen that the XOR null strain and the dual XOR/AO strains have a greatly reduced lifespan when compared with the type strain. The mean lifespan was approximately 15.9 \pm 2.4 days for maroon-like and 29.7 \pm 7.1 days for *ry*⁵⁰⁶ strains in comparison with 49.2 \pm 10.4 days for the wild type strain.

3.5. Effect of heat and cold shock on *Drosophila melanogaster* strains.

In the present study, the survivorship of wild type and mutant strains when they were exposed to heat and cold shock was investigated.

3.5.1. Effect of 25, 37 and 39°C on *Drosophila melanogaster* strains.

As expected all strains had 100% survival at 25°C. The mean percentage survival for all strains after the 37 and 39°C heat shock at 1, 2, 4 and 6 hours can be seen in figure 61. The temperatures and procedures used were according to that described by Gong and Golic (2005). All strains had no effect when were exposed to heat shock at 37°C for 2 hours. All the Canton-S flies survived at 4 hours but had a relatively high mortality rate at 6 hours (approximately 50%) at 37°C. The rosy strain had a significantly higher mortality rate after four hours (approximately 30%) and after six hours was approximately 60%. The heat shock at 37°C had a significant effect on the mortality of maroon-like strains; the survival rate of *mal-1* and *mal-f1* strains was approximately 40% and 37% respectively at 4 hours and all the flies were dead at 6 hours. At the highest temperature of 39°C at four and six hours found none of maroon-like strains survived. The heat shock at 39°C results showed that the *ry*⁵⁰⁶ strain had a significantly reduced percentage survival after 4 and 6 hours compared to the wild type Canton-S strain (shown figure 61). The rosy mutant strain at two hours at 39°C appears to have a low percentage survival (approximately 60%) and *mal-1* and *mal-f1* strains at the same duration appear to have a highly significant death levels with mortality being approximately 85% when, compared with Canton-S strain (approximately 20%).

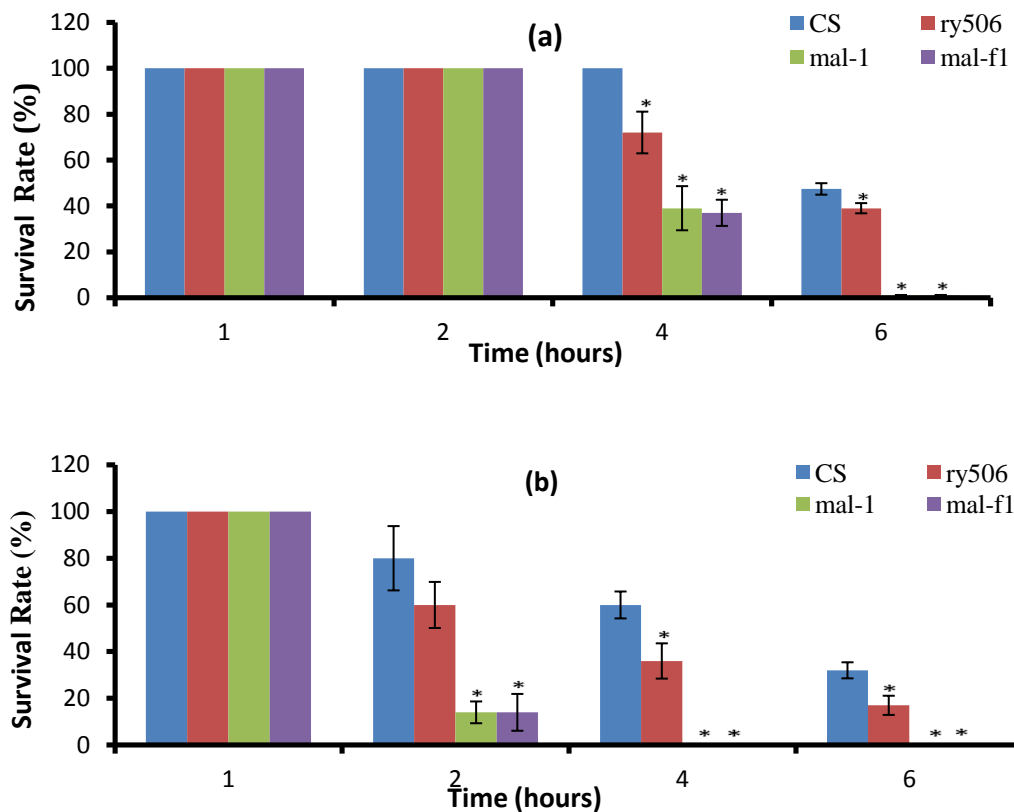


Figure 61: The survival percentage of *Drosophila* strains exposed to heat shock for different durations at 37 and 39°C temperature.

(* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value. (a) at 37°C. (b) at 39°C.

Statistical tests were carried out to check whether the effect of the heat shock on *Drosophila melanogaster* strains were significantly different. Table 33 presented the χ^2 values between all strains.

Table 33: Statistical difference in survival rate between wild type and mutant strains after exposure to the heat shock at 37 and 39°C.

Temperature degree and χ^2 value	Strain				
	Canton-S			<i>ry</i> ⁵⁰⁶	
	<i>ry</i> ⁵⁰⁶	<i>ma-l</i>	<i>mal-f1</i>	<i>mal-l</i>	<i>mal-f1</i>
37°C at 4 hours $\chi^2 =$	4.84*	26.76*	28.22*	22.75*	21.86*
37°C at 6hours $\chi^2 =$	5.10*	37.98*	39.01*	35.31*	37.01*
39°C at 2hours $\chi^2 =$	2.86	36.94*	35.80*	29.34*	28.11*
39°C at 4hours $\chi^2 =$	6.00*	47.80*	48.17*	42.97*	41.99*
39°C at 6hours $\chi^2 =$	4.59*	59.96*	61.10*	53.95*	52.77*

(* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

This indicated MFE-null strains were less able to survive at elevated temperature than MFE-active strains.

3.5.2. Effect of 0 and -3 °C cold shock on *Drosophila melanogaster* strains.

The mean percentage survival for all wild type and mutant strains when exposed to cold shock temperatures (0 and -3°C) for different periods (1, 2, 4, and 6 hrs) is illustrated in the following graph (Figure 62). There was no significant differences observed when the various strains were exposed to cold shock at 0 and -3°C for 1 hr. Canton-S strain had a steady decreased in percentage survival rate from ~90% to ~40% between 1 to 6 hours and all flies were dead when exposed to cold shock 0°C after 6 hours. For the period from 2 to 6 hours the rosy and *ma-l* strains had a higher significant mortality rate being ~ 80% of rosy strain at 6 hours and all *ma-l* flies were dead when exposed to 0°C. At -3°C cold shock temperature the mutant strains (*ry*⁵⁰⁶ and *ma-l*) had a highly significant different mortality rate for *ry*⁵⁰⁶ after 4 hours (approximately 98%) and all flies were dead after 6 hours. All *ma-l* strains were dead after 4 hours at -3°C (Figure 62).

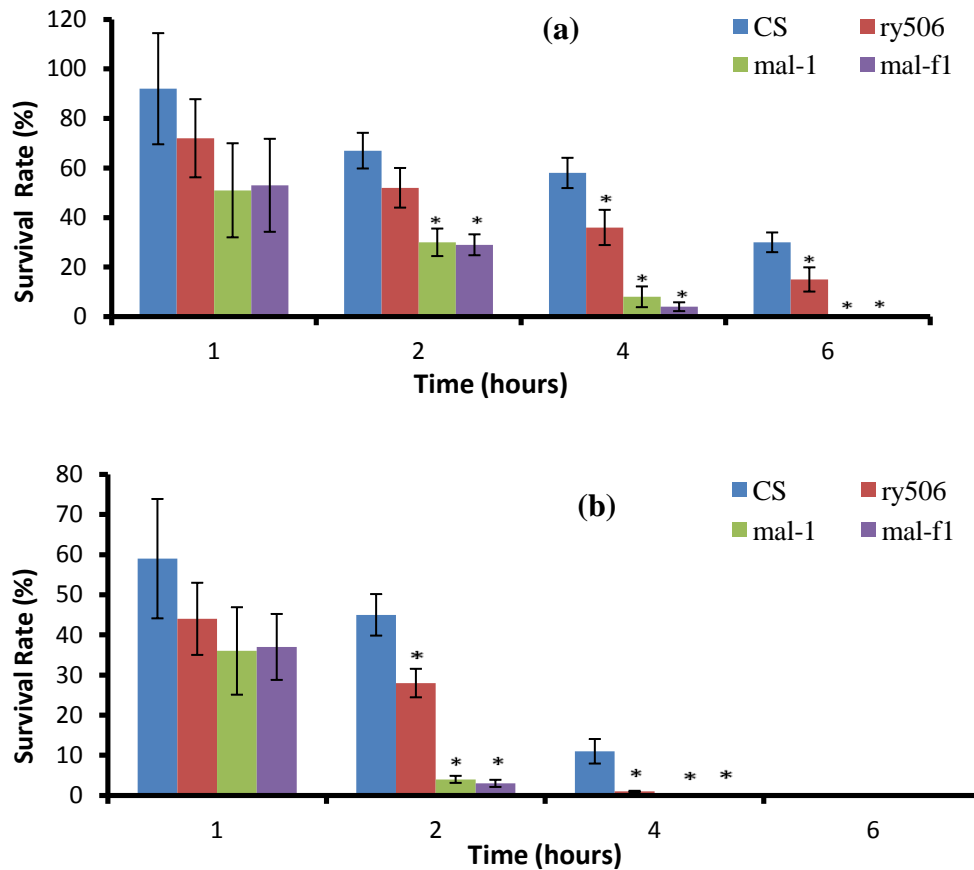


Figure 62: The survival percentage of *Drosophila* strains exposed to cold shock for different durations at 0 and -3°C.

(* $P < 0.05$ by χ^2 test) indicates when a value is significantly different from the control value. (a) at 0°C. (b) at -3°C.

Statistical tests were carried out to check whether the effect of the cold shock on *Drosophila melanogaster* strains were significantly different. Table 34 presented the χ^2 values between all strains. This indicated MFE-deficient strains were less able to survive cold shock than MFE-active strains.

Table 34: Statistical difference in survival rate between wild type and mutant strains after exposure to the cold shock at 0 and -3°C.

Temperature degree and χ^2 value	Strain				
	Canton-S			<i>ry</i> ⁵⁰⁶	
	<i>ry</i> ⁵⁰⁶	<i>ma-l</i>	<i>mal-f1</i>	<i>mal-1</i>	<i>mal-f1</i>
0°C at 2 hours $\chi^2 =$	3.07	10.43*	9.79*	8.02*	8.70*
0°C at 4 hours $\chi^2 =$	6.09*	37.97*	39.02	24.34*	25.12*
0°C at 6 hours $\chi^2 =$	11.41*	61.44*	60.71*	51.27*	50.98*
-3°C at 2 hours $\chi^2 =$	9.95*	49.05*	47.87*	27.42*	26.72*
-3°C at 4 hours $\chi^2 =$	5.33*	37.40*	36.98*	1.04	1.10

(* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

3.6. Bioinformatics analysis of *Drosophila molybdenum* cofactor sulphurase (MCS) gene.

In order to identify mutations in the MCS gene in *mal-1* and *mal-fl* strains bioinformatic analysis was performed in order to map intron/exon boundaries and to design PCR primers. This was necessary as there has been no publication detailing these. As the *Drosophila* genome had been completely sequenced (Adams *et al.*, 2000) the gene sequence was available on the Genbank database (AC011758). Along with the complete gene, the cDNA sequence was also found and the two were then compared using bioinformatics software (see section 2.9.1). This showed that the coding sequence of the gene was split over 4 exons. The intron/exon boundaries were also deduced. Once the boundaries had been established primer pairs were designed in order to clone the complete sequence. Nine primer pairs were selected forming an overlapping contig covering the entire gene (Figure 63).

-647 gatgtaaatcgataaatacagctgtatgtgtaggctatogctagatgtgaaacgaatttaattgttctgtagtgtaaaaaacattttataaattgtttataaataaagattgttagtttccaaaaattattgtcacaataatggccaattagccattagcaaacattttcctaacaaccaattgtttgccaacactcagaacatatgtttgaatagccatgcaaaagtcacacagactagttaata

-404 cagtcaaatgttagtttaaaattctatgcgaattcagagtttaataattggaagaagatttggttaaaatgctacatctataa**atttggtgagcagtg**gagaatatgatataatattacaataagcaacataatgcacaagaagcttaataaattgagcttcgtaaaattgtgtagggtgagta

-161 agttcaagagaagtcacaaatgctcaactgaacgtgatagttgtatgtatactgtccgtggcttt**ttccagCGAAGAAGATGTG**CGGCAACAAATGCAAGCCGGTCGAAATAAATAAGCCCGCTTTGCGACCAACACGAATAGTTAGTTCCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAATAGACGCGGAATTTCTAGATTGGCCAGTAAGtgga
MetThrSerTyrArgProGluPheSerAlaSerGluGlnSerGlnIleAspAlaGluPheSerArgLeuAlaSerAs (26)

+161 gaacacatggtgaaataccacagcgcaaacagataaacacctaactatccgtctatagaacaggcaacAAGAGTGTTTACCTGGATCATGCGGGGCCACCTCTCTATCGCGAGAGCCAGGTGACGGCCGGCCGGGCAACTGCAGCGCAATGTCATCTGCAATCCGCACACCTCGCCGCTCACCGGCGACTTCGTCGACAGGTG**CGATTCAAGtgagtttcacggtg**ccacagagccaaaac
nLysSerValTyrLeuAspHisAlaGluThrThreuleTyrAlagluSerGlnValThrAlaAlaAlaGluGlnLeuGlnArgAsnValIleCysAsnArgHisThrCysArgLeuThrGlyAspPheValAspGlnValArgPheLy (75)

+325 aaccattotococctgtcaattgggttgocottgtgtggcgcttttattataaattattattgtgcataatagcaataatcgttaaaatactctattgtcgctgtctattctctcatttctgttggttaagttgttttagtaaaactcttatcatatacacttttttagAATACTGGGAATTCCTTAACACCACCGCCGAAGATTACCATGTGATATTACGGCGAATGCCACCGCTGCTTTGTCCCT
sIleLeuGluPhePheAsnThrThrAlaGluAspTyrHisValIlePheThrAlaAsnAlaThrAlaAlaLeuSerLe (101)

+568 GGTGCGGAAAACTTTGACTTTGGATCTCTGGTGAAGTTTCACTTCTGCCAGGAGAACACACCTCAGTGCTGGGAATGCGGGAAAGGGTCGGGAAAAACGGTATATACATGCTGAGGGAGAATGAAATCTCTGCGGAAAGCACAAGGCAACGGGAAAGTTACAGAGGTGTCCGGAAAAACAGGAACTCCTTGCTGACCTTTTCGGCCCAATGCAACTTTAGTGGTTACAAAAATCCCACT
sValAlaGluAsnPheAspPheGlySerSerGlyGluPheHisPheCysGlnGluAsnHisThrSerValLeuGlyMetArgGluArgValArgGluAsnGlyIleTyrMetLeuArgGluAsnGluIleSerGlyGlyLysHisLysAlaAsnGlyLysValHisGluValSerGlyLysThrGlyAsnSerLeuLeuThrPheSerAlaGlnCysAsnPheSerGlyTyrLysIleProLe (182)

+811 GGAAGTCATTGAGCAATCCAAATCGATGCTTTGGCCAAAGCCGGAAGAGCTTTGGAGCTCTTTGGGAGAGAAGAAGAATATGCACAACGATTACTATATCTGCTGGATGCGGCGTCTTTTGTGGCCACAGTCCGTTGGATCTGCAAAAGTATAGACCAGATTACGTGTGCCTTAGTTTCTACAAGATCTTCGGTTATCCACGGGCGTGGGAGCCCTGCTCGTGAGTCGACGAGG
uGluValIleGluGlnIleGlnIleAspGlyLeuAlaLysProGlyLysGluLeuTrpSerSerLeuGlyGluLysLysLysAsnMetHisAsnAspTyrTyrIleCysLeuAspAlaAlaSerPheValAlaThrSerProLeuAspLeuGlnLysTyrArgProAspTyrValCysLeuSerPheTyrLysIlePheGlyTyrProThrGlyValGlyAlaLeuValValSerArgArgGl (263)

+1054 **ACCGAGGCTCTCCAGAG**SCGCCGCTTTTGGCGGTGTACCATCACTACGCATATCCGATGCCATGGATTACAGCTGAGAGAGACCTTCCATCAGCGCTACGAGGACGGCACCT**TCGCCANTTCCTCTCCATTGT**CGGGTTGCTCGAGGGCTTTTGAACGCTGGAGAGATTGGTGCCCAAGACGGACGAGTTAGCACCATGGAGAGGATTTCAGtgtagataatgataagctga
yAlaGluValPheGlnLysArgArgPhePheGlyGlyGlyThrIleAsnTyrAlaTyrProHisAlaMetAspTyrGlnLeuArgGluThrPheHisGlnArgTyrGluAspGlyThrLeuProPheLeuSerIleValGlyLeuLeuGluGlyPheArgThrLeuGluArgLeuValProArgThrAspGluPheSerThrMetGluArgIleSerAr (333)

+1540 tgacttcagccttattttccaatcattgtattttcagACACGCTCTTGGTCTGCGCAATATCTGGAGGATCAGCTGCGCCAGCTGCATATCCCAATGGTGAGCCCTCTGTGAAGCTGTACAATAAAGTGGGTACCAAGACAAATCCCGGCAAGGTGGCATCGTGGCTTTAACGTGCGCACTGAATCGGGTTCGTTTGTGGGATTCGGCGAGATCGCTGTGTGGTCTCTGCACGGAA
gHisvalPheGlyLeuAlaLysTyrLeuGluaspGlnLeuArgGlnLeuHisHisProAsnGlyGluProLeuValLysLeuTyrAsnLysValGlyTyrGlnAspLysSerArgGlnGlyIleValAlaPheAsnValArgThrGluSerGlySerPheValGlyPheGlyGluIleAlaCysValAlaAlaLeuHisGlyI (414)

+1783 **TACTCTCGGACTGGTTGCTTCTGCAACATCGGAGCTCGCAATATTACCTAGGCCTCGATGAAGATGCTTTGGATGCGATT**TACAAGCGAGCAGGTGCGATATGCGCGGACTACTTTGATCTGATCATGAGCAGCGGACGGGACGGGTACGGGTATCTTTTGGCTATAT**TCGCTCTTTCAGGAC**TAGACAAGCTGCTCCAGATGCTGCGCTCCAGTTATTTTGGCAACCAAGCCGCTGC
leLeuLeuArgThrGlyCysPheCysAsnIleGlyAlaCysGlnTyrTyrLeuGlyLeuAspGluAspAlaLeuAspAlaIleTyrArgArgAlaGlyArgIleCysGlyAspTyrPheAspLeuIleAspGlyGlnProThrGlyAlaValArgValSerPheGlyTyrMetThrThrIleGlnAspValAspLysLeuLeuGlnMetLeuArgSerSerTyrLeuAlaThrArgProLeuG (495)

+2026 AGCGATTTCAGTTTCATCGAGGACGAGCGGAGCA**ATTGCGCGGTGTCTGA**AGCGGAGTCCAGCTCTTGAGGCCAAACTATTGCAGATGGCCATATATCCGTTAAAGCTCTTGCGCGCTTTAAGATCGAATTACCGGGCTCCTGGCCATTGACGGATCAAGGTTTGAATATGACCGTGAGTGGATGCTGGACATGAATGGCATGGCGTTGACCCAGAAACGCTGCACGGAGTTGT
InArgIleGlnPheIleGluGluGlnAlaGluGlnLeuProProLeuLeuLysGluArgValGlnLeuLeuArgProLysLeuLeuGlnMetAlaIleTyrProValLysSerCysAlaAlaPheLysIleGluLeuProGlySerTrpProLeuThrAspGlnGlyLeuLysTyrAspArgGluTrpMetIleValAspMetAsnGlyMetAlaLeuThrGlnLysArgCysThrGluLeuC (576)

+2269 GCCTAATTAGGCCAGTGATTAAAGTTGACCAATTGGAGCTCGAGTTTGAGAAAAATCCACATCTCGTGCCCCCTTCTCTGGATGACCGGACCGGACACAGCAAAATGCG**TAAGCAAGGTGTGCGGACAG**CCCGTGGAAGGTTTGGATTGCGGCGATAGGGTTGCCAGTGTTAAGCGAAACCTCGGCATGGAGGGCCCTCGTTTGCTTGACAATCGGGCCAGAGAACTCTTCCA
TGCCTAAGCAAGGTGTGC
AATGCGTAAGCAAGGTGTGCGGAC
ysLeuIleArgProValIleLysValAspGlnLeuGluLeuGlnPheGlyGluAsnSerThrIleSerValProLeuSerLeuAspAspGlnAlaAlaAspThrAlaLysCysValSerLysValCysArgGlnProValGluGlyLeuAspCysGlyAspArgValAlaGlnTrpLeuSerGluAsnLeuGlyMetGluGlyLeuArgLeuLeuArgGlnSerGlyGlnArgAsnSerSerL (638)

+2512 AGGATCAGCAGAGCTTAAGTCTGTTTAATCAGGCCAGTTCTTGCTGCTTAAACAGTCTTCCGTGCGATCGCTTCAATTTGAAGAGCCCTCGACGAGACTGTGGATCGTTTTTCGGGCAATATCATCATCGACCGGCGAGTGCCTTTTGGAGAGCTTACCTACAAGGCCCTGTCCATTGGGGGAATCCAATTTCAAGTGAGGGTCCCTGCCACGCTGCGCATGATCTGCATTAAACAAA
ysAspGlnGlnLysLeuserLeuValAsnGlnAlaGlnPheLeuLeuLeuAsnLysSerSerValArgSerLeuGlnPheGluGluAspLeuAspGluThrValAspArgPheArgAlaAsnIleIleIleAspThrGlySerAlaPheGluGluLeuThrTyrLysAlaLeuSerIleGlyGlyIleGlnPheGlnValGluGlyProCysGlnArgCysAspMetIleCysIleAsnGlnA (729)

+2755 GGACAGCGCAACGCTCGCCAGAACTTTGACCACCATATCCGCCTGCAAGAGGTGCGATCGGATTCGGCATCTACATCACTAGGATCCCCAGGACACAAAAGAACTGGAACCAAGGAGCAACACATGACCTGCGGCGATGTTGCTCTTGTGGAATAAGCTCACTTTATCATCACTTCAGCGTGGTTTTCTTCGTTCTTTGGTTCGTTGACCCGAAATGTTTCTATTGTACATAGTTTATG
rgThrGlyGluArgSerProGluThrLeuThrThrIleSerArgLeuGlnLysGlyArgMetArgPheGlyIleTyrIleThrArgIleProGlnAspThrLysGluLeuGluProLysGluGlnHisMetThrCysGlyAspValValLeuValGluStp (782)

+2998 CACAATATTCTACTAAGTAATCTCTATTACGTTGCATCAGTTTCGCACCTTAGTTTGTGTTTTTAAGTACTTCTGAAATAGGAATACGCAAACTTTAAAGAAACATTCCAGGATAGCTTAAAGTTCAAAGTAACCGGATACCTCTTGCCAGTGCTTAAAGCCTTTAAAGGCCTTAATTGCAAAATAAACACATTGAAATCGatgtatatacttgagttcttaggaagggttaatcgaaact

+3236 aaataacgcaacgctgataaataatgcgaattgaatccaggcgccatttgaagaagtgcagcagcggctaccaacgaatcatggaagcaaatattgacagaagttgcttaacatcaataatgataataatgaataaaagcatcgcgatataacttcatacacaacatcataaaataatgattatatttcttgaactgttaaagctctataaattaccaaggctt

Figure 63: Complete gene sequence for the MCS gene in *Drosophila melanogaster* showing deduced amino acid sequence, exonic sequence shown in upper case black letters and intronic sequence shown in lower case black letters.

Primer pairs are indicated in different colours and are presented in table 20 in section 2.9.3. The nucleotides are numbered from the transcription initiation site (position +1).

3.6.1. Design of PCR primers for the amplification of the *Drosophila molybdenum* cofactor sulpharase gene.

Primers sequences were selected by using the computer programs as described in materials and methods section 2.9.1 to find primer pairs that would hybridise to regions located within 1 kb of each other.

3.6.2. Optimisation of the PCR conditions for amplification of *Drosophila molybdenum* cofactor sulpharase gene.

Once the PCR primers were designed PCR optimisation was achieved by using the basic components as listed in materials and methods (section 2.9.3), varying one component at a time. The basic variable components were the temperature the PCR was carried out at, the concentration of the primers and the concentration of the $MgCl_2$. Table 35 shows a summary of the conditions used and the results obtained using DNA isolated from the wild type strain (Canton-S) of *Drosophila melanogaster*. The optimal conditions for each primer pair are highlighted in yellow. An agarose gel image showing the successfully amplified products for each of the primer pairs is shown in figure 64.

Table 35: Summary of the experimental conditions and outcomes of the PCR of the *Drosophila* MCS gene.

Primers set	Annealing temperatures °C	Primer conc.*	MgCl ₂ conc.*	Fragments Generated
DMCS1	50,52,54,56,58	0.5 pM	2 mM	Several fragments
	50,52,54,56,58,60,62	0.5 pM	3 mM	Several fragments
	57	0.5 pM	1.5 mM	570 bp fragment
DMCS2	47,49,51,53,55,57,60,62	0.5 pM	1.5 mM	No fragments
	48,50,52,54,56,58,60,62	0.5 pM	3 mM	Several fragments
	47,49,51,53,55,57	0.5 pM	2 mM	Several fragments
	47,49,51,53,55,57,60,62	0.5 pM	2.5 mM	Several fragments
	57,58,59,60,61,62	0.5 pM	3.5 mM	Several fragments
	52,53,54,55,56	0.5 pM	1.5 mM	Several fragments
	47,49,51,53,55,57	0.5 pM	1.5 mM	Several fragments
	47,49,51,53,55,57	0.5 pM	3 mM	Several fragments
	55	0.25 pM	1.5 mM	730 bp fragment
DMCS3	50,52,54,56,58	0.5 pM	2 mM	Several fragments
	59	0.5 pM	1.5 mM	780 bp fragment
DMCS4	55	0.5 pM	1.5 mM	1100 bp fragment
DMCS5	57	0.5 pM	1.5 mM	660 bp fragment
DMCS6	55	0.5 pM	1.5 mM	980 bp fragment
DMCS7	47,49,51,53,55,57,60,62	0.5 pM	1.5 mM	Several fragments
	48,50,52,54,56,58,60,62	0.5 pM	3 mM	Several fragments
	47,49,51,53,55,57	0.5 pM	2 mM	Several fragments
	47,49,51,53,55,57,60,62	0.5 pM	2.5 mM	Several fragments
	57,58,59,60,61,62	0.5 pM	3.5 mM	Several fragments
	52,53,54,55,56	0.5 pM	1.5 mM	Several fragments
	47,49,51,53,55,57	0.5 pM	1.5 mM	Several fragments
	47,49,51,53,55,57	0.25 pM	2 mM	Several fragments
	55	0.25 pM	1.5 mM	610 bp fragment
DMCS8	55	0.5 pM	1.5 mM	440 bp fragment
DMCS9	50,52,54,56,58,60,62	0.5 pM	1.5 mM	Several fragments
	53	0.25 pM	1.5 mM	880 bp fragment

This table shows the optimisation process for primer sets from DMCS1 to DMCS9. It illustrates the temperatures used with each set of conditions and the optimised outcomes from each set of conditions used, indicated by yellow colour. *concentration.

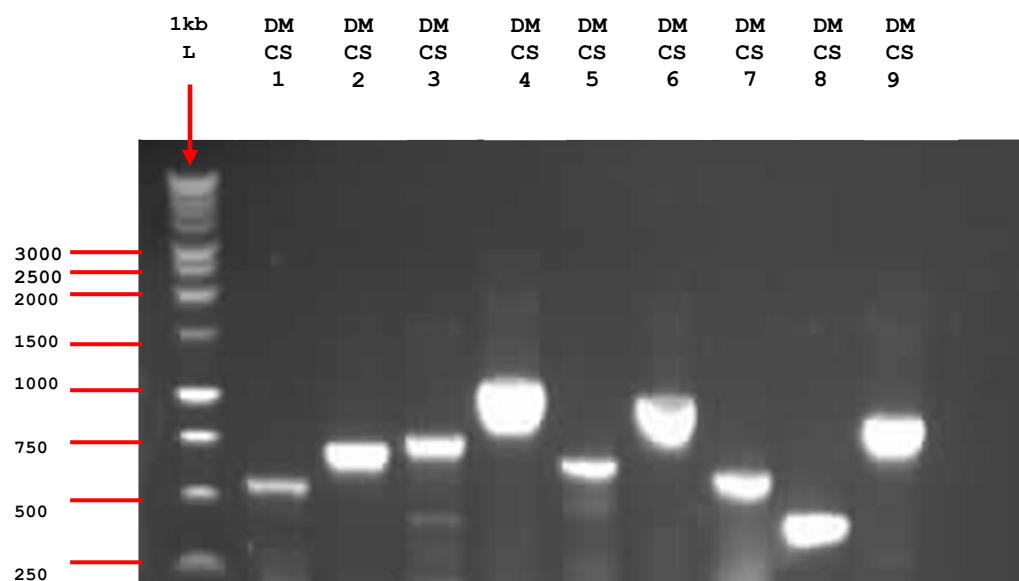


Figure 64: Agarose gel electrophoresis of products obtained by PCR amplifying different regions of Canton-S *Drosophila* molybdenum cofactor sulfurase gene. DMSC1-9 sets used from Table 27.

Once the optimal conditions had been obtained for each of the nine primer sets, to confirm that the correct region of the *Drosophila* genome had been amplified the nine products obtained were sequenced in both the forward and reverse directions. The DNA sequencing results from the nine amplified products were used to produce a contig of the wild type sequence. The contig sequence produced was compared to the known GenBank sequence. The coding region of the Canton-S wild type sequence was 99% identical to that of the known GenBank sequence (AC011758 *Drosophila melanogaster*, chromosome X, region 19C-19E, BAC clone and AF162681 *Drosophila melanogaster* maroon-like protein (*mal*) gene, complete). A number of nucleotide changes were identified in the non-coding and coding regions of the gene (Figure 65). This confirmed that the region amplified was the *Drosophila* MCS gene. The nucleotide sequence variations between Canton-S (wild type) DNA and database AC01178 and AF162681 were in intron 1 (IVSI-93C>T, IVSI-135A>T and IVSI-151A>G), exon 3 (c.260T>A, c.808C>A and c.1196G>A) and exon 4 (c.1884A>C, c.2483A>C, c.2143C>A, c.2289G>T, c.2567C>T and c.2890C>T) (see figure 51).

Figure 65: Comparison of the nucleotide sequence of the Canton-S *D. melanogaster* strain MCS gene (CSDMCS) obtained in this study with those on GeneBank.

Nucleotide sequence alignment of the Canton-S *D. melanogaster* MCS gene which was obtained from PCR products and compared with AC011758 *D. melanogaster* chromosome X region 19C-19E, BAC clone AF162681 *D. melanogaster* maroon-like protein (*ma-l*) gene were aligned using CLUSTAL X program (Larkin *et al.*, 2007). The MSC gene exonic sequences are highlighted in different colours and intronic sequence shown in lower case letters. The sequences in upper case italics are 5' and 3' untranslated sequences in the mRNA. The differences nucleotides highlighted in red text. AC011758 = *D. melanogaster* chromosome X region 19C-19E BAC clone. AF162681 = *D. melanogaster* maroon-like protein (*ma-l*) gene.

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CSDMCS      (-179)  acccagaaacgctgcacggagttgtgcaagttcaaagagaagtcaaagcaaatgttcaactgaacgtgatatgtactgtccgtggttttctccagCGAAGAAGAGTATGGCGCAACAAATGCAAGCCGGTCGCAAAAT
AC011758    (-179)  acccagaaacgctgcacggagttgtgcaagttcaaagagaagtcaaagcaaatgttcaactgaacgtgatatgtactgtccgtggttttctccagCGAAGAAGAGTATGGCGCAACAAATGCAAGCCGGTCGCAAAAT
AF162681    (-179)  acccagaaacgctgcacggagttgtgcaagttcaaagaaaagtcaaagcaaatgttcaactgaacgtgatatgtactgtccgtggttttctccagCGAAGAAGAGTATGGCGCAACAAATGCAAGCCGGTCGCAAAAT
*****
CSDMCS      (-45)   AAATAAAGCCCGATTTCGAGCCAACACGAATAGTTAGTTCCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAAAATAGACGCGGAATTCTCTAGATTGGCCAGTAAGtgaggagaacccatggtgaaatac
AC011758    (-45)   AAATAAAGCCCGCTTTTCGAGCCAACACGAATAGTTAGTTCCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAAAATAGACGCGGAATTCTCTAGATTGGCCAGTAAGtgaggagaacccatggtgaaatac
AF162681    (-45)   AAATAAAGCCCGATTTCGAGCCAACACGAATAGTTAGTTCCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAAAATAGACGCGGAATTCTCTAGATTGGCCAGTAAGtgaggagaacccatggtgaaatac
*****
CSDMCS      (+243)  ccagcgcaaacagataacgaacctaactatccgtctatcgaacaggcaaCAAGAGTGTTTACCTGGATCATGCGGGCACCCTCTCTATGCCGAGAGCCAGGTGACGGCCGCCGCGGAGCAACTGCAGCGCAATGTCATCTGCA
AC011758    (+243)  ccagcgcaaacagataacgaacctaactatccgtctatcgaacaggcaaCAAGAGTGTTTACCTGGATCATGCGGGCACCCTCTCTATGCCGAGAGCCAGGTGACGGCCGCCGCGGAGCAACTGCAGCGCAATGTCATCTGCA
AF162681    (+243)  ccagcgcaaacagataacgaacctaactatccgtctatcgaacaggcaaCAAGAGTGTTTACCTGGATCATGCGGGCACCCTCTCTATGCCGAGAGCCAGGTGACGGCCGCCGCGGAGCAACTGCAGCGCAATGTCATCTGCA
*****
CSDMCS      (+387)  ATCCGCACACCTGCCGCCTCACCGGCGACTTCGTTCGACCAAGTGCGATTCAAGtgagttcatccgtgtccacgagcgaacaaacaccattctccccctgtcattgggttgcccttggtgtggcgcttttattataaattatttatg
AC011758    (+387)  ATCCGCACACCTGCCGCCTCACCGGCGACTTCGTTCGACCAAGTGCGATTCAAGtgagttcatccgtgtccacgagcgaacaaacaccattctccccctgtcattgggttgcccttggtgtggcgcttttattataaattatttatg
AF162681    (+387)  ATCCGCACACCTGCCGCCTCACCGGCGACTTCGTTCGACCAAGTGCGATTCAAGtgagttcatccgtgtccacgagcgaacaaacaccattctccccctgtcattgggttgcccttggtgtggcgcttttattataaattatttatg
*****
CSDMCS      (+531)  tgcatatagcaatatcgtaaaatatccttatgtcgctgcttatcttctcatttgtggttaagtgttttttagtaaaacttctattcatatacacttttttagAATACTGGAATTCTTTAAACACCACCGCGGAAGATTACCATG
AC011758    (+531)  tgcatatagcaatatcgtaaaatatccttatgtcgctgcttatcttctcatttgtggttaagtgttttttagtaaaacttctattcatatacacttttttagAATACTGGAATTCTTTAAACACCACCGCGGAAGATTACCATG
AF162681    (+531)  tgcatatagcaatatcgtaaaatatccttatgtcgctgcttatcttctcatttgtggttaagtgttttttagtaaaacttctattcatatacacttttttagAATACTGGAATTCTTTAAACACCACCGCGGAAGATTACCATG
*****
CSDMCS      (+675)  TGATATTACGGCGAATGCCACCGCTGCTTTGTCCCTGGTGGCGGAAACCTTTGACTTTGGATCCTCTGGTGAGTTTCACCTTCTGCCAGGAGAACCACACCTCAGTGCTGGGAATGCGGGAAAGGGTCCGGGAAACGGTATAT
AC011758    (+675)  TGATATTACGGCGAATGCCACCGCTGCTTTGTCCCTGGTGGCGGAAACCTTTGACTTTGGATCCTCTGGTGAGTTTCACCTTCTGCCAGGAGAACCACACCTCAGTGCTGGGAATGCGGGAAAGGGTCCGGGAAACGGTATAT
AF162681    (+675)  TGATATTACGGCGAATGCCACCGCTGCTTTGTCCCTGGTGGCGGAAACCTTTGACTTTGGATCCTCTGGTGAGTTTCACCTTCTGCCAGGAGAACCACACCTCAGTGCTGGGAATGCGGGAAAGGGTCCGGGAAACGGTATAT
*****

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CSDMCS (+819) ACATGCTGAGGGAGAATGAAATCTCTGGCGGAAAGCACAAGCGAACGGGAAAGTTACAGAGGTGTCCGGAACAGGAAACTCCTTGCTGACCTTTTCGCGCCCAATGCAACTTTAGTGTTACAAAATCCCACTGGAAGTCA
AC011758 (+819) ACATGCTGAGGGAGAATGAAATCTCTGGCGGAAAGCACAAGCGAACGGGAAAGTTACAGAGGTGTCCGGAACAGGAAACTCCTTGCTGACCTTTTCGCGCCCAATGCAACTTTAGTGTTACAAAATCCCACTGGAAGTCA
AF162881 (+819) ACATGCTGAGGGAGAATGAAATCTCTGGCGGAAAGCACAAGCGAACGGGAAAGTTACAGAGGTGTCCGGAACAGGAAACTCCTTGCTGACCTTTTCGCGCCCAATGCAACTTTAGTGTTACAAAATCCCACTGGAAGTCA

CSDMCS (+963) TTGAGCAAATCCAAATCGATGGCTTGGCCAAGCCGGGAAAAGAGCTTTGGAGCTCTTTGGGAGAGAAGAAGAATAATGCACAACGATTACTATATCTGCCTGGATGCGGCGCTCTTTTGTGGCCACCAGTCCGTTGGATCTGC
AC011758 (+963) TTGAGCAAATCCAAATCGATGGCTTGGCCAAGCCGGGAAAAGAGCTTTGGAGCTCTTTGGGAGAGAAGAAGAATAATGCACAACGATTACTATATCTGCCTGGATGCGGCGCTCTTTTGTGGCCACCAGTCCGTTGGATCTGC
AF162881 (+963) TTGAGCAAATCCAAATCGATGGCTTGGCCAAGCCGGGAAAAGAGCTTTGGAGCTCTTTGGGAGAGAAGAAGAATAATGCACAACGATTACTATATCTGCCTGGATGCGGCGCTCTTTTGTGGCCACCAGTCCGTTGGATCTGC

CSDMCS (+1107) AAAAGTATAGACCAGATTACGTGTGCCTTAGTTTCTACAAGATCTTCGGTTATCCACGGGCGTGGGAGCCCTGCTCGTGAGTCGACGAGGAGCCGAGGTCCTCCAGAAGCGCCGCTTTTGTGGCGGTGGTACCATCAACTAC
AC011758 (+1107) AAAAGTATAGACCAGATTACGTGTGCCTTAGTTTCTACAAGATCTTCGGTTATCCACGGGCGTGGGAGCCCTGCTCGTGAGTCGACGAGGAGCCGAGGTCCTCCAGAAGCGCCGCTTTTGTGGCGGTGGTACCATCAACTAC
AF162881 (+1107) AAAAGTATAGACCAGATTACGTGTGCCTTAGTTTCTACAAGATCTTCGGTTATCCACGGGCGTGGGAGCCCTGCTCGTGAGTCGACGAGGAGCCGAGGTCCTCCAGAAGCGCCGCTTTTGTGGCGGTGGTACCATCAACTAC

CSDMCS (+1251) GCATATCCGCATGCCATGGATTACCAGCTGAGAGAGACCTTCCATCAGCGCTACGAGGACGGCACCCCTGCCATTCTCTCCATTGTCGGGTTGCTCGAGGGCTTTTCGAACGCTGGAGAGATTGGTGCCCGAGAACGGACGAGTT
AC011758 (+1251) GCATATCCGCATGCCATGGATTACCAGCTGAGAGAGACCTTCCATCAGCGCTACGAGGACGGCACCCCTGCCATTCTCTCCATTGTCGGGTTGCTCGAGGGCTTTTCGAACGCTGGAGAGATTGGTGCCCGAGAACGGACGAGTT
AF162881 (+1251) GCATATCCGCATGCCATGGATTACCAGCTGAGAGAGACCTTCCATCAGCGCTACGAGGACGGCACCCCTGCCATTCTCTCCATTGTCGGGTTGCTCGAGGG- TTTTCGAACGCTGGAGAGATTGGTGCCCGAGAACGGACGAGTT

CSDMCS (+1395) TAGCACCATTGGAGAGGATTTCCAGtgattagataaatgataagtctgatgacttcagccttattttccaatcattgtattttcagACACGCTTTTGGTCTGGCCAAAGTATCTGGAGGATCAGCTGCGCCAGCTGCATCATCCC
AC011758 (+1395) TAGCACCATTGGAGAGGATTTCCAGtgattagataaatgataagtctgatgacttcagccttattttccaatcattgtattttcagACACGCTTTTGGTCTGGCCAAAGTATCTGGAGGATCAGCTGCGCCAGCTGCATCATCCC
AF162881 (+1395) TAGCACCATTGGAGAGGATTTCCAGtgattagataaatgataagtctgatgacttcagccttattttccaatcattgtattttcagACACGCTTTTGGTCTGGCCAAAGTATCTGGAGGATCAGCTGCGCCAGCTGCATCATCCC

CSDMCS (+1539) AATGGTGAGCCCTCGTGAAGCTGTACAATAAAGTGGGCTACCAAGACAAATCCCGGCAGGGTGGCATCGTGGCCCTTAAACGTGCGCACTGAATCGGGTTCGTTTGTGGGATTTCGCGAGATCGCCTGTGTGGCTGCTCTGCA
AC011758 (+1539) AATGGTGAGCCCTCGTGAAGCTGTACAATAAAGTGGGCTACCAAGACAAATCCCGGCAGGGTGGCATCGTGGCCCTTAAACGTGCGCACTGAATCGGGTTCGTTTGTGGGATTTCGCGAGATCGCCTGTGTGGCTGCTCTGCA
AF162881 (+1539) AATGGTGAGCCCTCGTGAAGCTGTACAATAAAGTGGGCTACCAAGACAAATCCCGGCAGGGTGGCATCGTGGCCCTTAAACGTGCGCACTGAATCGGGTTCGTTTGTGGGATTTCGCGAGATCGCCTGTGTGGCTGCTCTGCA

AC011758 (+1683) CGGAATACTCTGCGGACTGGTTGCTTCTGCAACATCGGAGCCTGCCAATATTACCTAGGCCTCGATGAAGATGCTTTGGATGCGATTATACAAGCGAGCAGGTGCGATATGCGGCGACTACTTTGATCTGATCGATGGACAGC
AF162881 (+1683) CGGAATACTCTGCGGACTGGTTGCTTCTGCAACATCGGAGCCTGCCAATATTACCTAGGCCTCGATGAAGATGCTTTGGATGCGATTATACAAGCGAGCAGGTGCGATATGCGGCGACTACTTTGATCTGATCGATGGACAGC
CSDMCS (+1683) CGGAATACTCTGCGGACTGGTTGCTTCTGCAACATCGGAGCCTGCCAATATTACCTAGGCCTCGATGAAGATGCTTTGGATGCGATTATACAAGCGAGCAGGTGCGATATGCGGCGACTACTTTGATCTGATCGATGGACAGC

CSDMCS (+1827) CGACGGGAGCGGTACGGGTATCTTTTGGCTATATGACAACATATTACGAGCCTAGACAGCTGCTCCAGATGCTGCGCTCCAGTTATTTGGCAACCAAGCCGCTGCAGCGGATTACGTTTCATCGAGGAGCAGGCGGAGCAATTG
AC011758 (+1827) CGACGGGAGCGGTACGGGTATCTTTTGGCTATATGACAACATATTACGAGCCTAGACAGCTGCTCCAGATGCTGCGCTCCAGTTATTTGGCAACCAAGCCGCTGCAGCGGATTACGTTTCATCGAGGAGCAGGCGGAGCAATTG
AF162881 (+1827) CGACGGGAGCGGTACGGGTATCTTTTGGCTATATGACAACATATTACGAGCCTAGACAGCTGCTCCAGATGCTGCGCTCCAGTTATTTGGCAACCAAGCCGCTGCAGCGGATTACGTTTCATCGAGGAGCAGGCGGAGCAATTG

CSDMCS (+1971) CCGCCGTTGCTGAAGGAGCGAGTCCAGCTCTTGAGGCCAAAATATTGCAGATGGCCATATATCCGGTAAAGTCTTGTGCCGCTTTAAGATCGAATTACCGGGCTCCTGGCCATTGACGGATCAAGGTTTGAATATGACCG
AC011758 (+1971) CCGCCGTTGCTGAAGGAGCGAGTCCAGCTCTTGAGGCCAAAATATTGCAGATGGCCATATATCCGGTAAAGTCTTGTGCCGCTTTAAGATCGAATTACCGGGCTCCTGGCCATTGACGGATCAAGGTTTGAATATGACCG
AF162881 (+1971) CCGCCGTTGCTGAAGGAGCGAGTCCAGCTCTTGAGGCCAAAATATTGCAGATGGCCATATATCCGGTAAAGTCTTGTGCCGCTTTAAGATCGAATTACCGGGCTCCTGGCCATTGACGGATCAAGGTTTGAATATGACCG

CSDMCS (+2115) TGAGTGGATGATCGTGGACATGAATGGAATGGCGTTGACCCAGAAACGCTGCACGGAGTTGTGCCATAATTAGGCCAGTGATTAAGTTGACCAATTGGAGTGCAGTTTGGAGAAAATCCACCATCTCGGTGCCCTTTCTCT
AC011758 (+2115) TGAGTGGATGATCGTGGACATGAATGGAATGGCGTTGACCCAGAAACGCTGCACGGAGTTGTGCCATAATTAGGCCAGTGATTAAGTTGACCAATTGGAGTGCAGTTTGGAGAAAATCCACCATCTCGGTGCCCTTTCTCT
AF162881 (+2115) TGAGTGGATGATCGTGGACATGAATGGAATGGCGTTGACCCAGAAACGCTGCACGGAGTTGTGCCATAATTAGGCCAGTGATTAAGTTGACCAATTGGAGTGCAGTTTGGAGAAAATCCACCATCTCGGTGCCCTTTCTCT

CSDMCS (+2259) **GGATGACCAGGCAGCCGACACAGCAAAATGTGTAAGCAAGGTGTGCCGACAGCCCGTGGAAGGTTTGGATTGCGGCGATAGGGTTGCCAGTGGTTAAGCGAAAACCTCGGCATGGAGGGCCTCCGTTTGCTTCGACAATCGG**

AC011758 (+2259) GGATGACCAGGCAGCCGACACAGCAAAATGCGTAAGCAAGGTGTGCCGACAGCCCGTGGAAGGTTTGGATTGCGGCGATAGGGTTGCCAGTGGTTAAGCGAAAACCTCGGCATGGAGGGCCTCCGTTTGCTTCGACAATCGG

AF162881 (+2259) GGATGACCAGGCAGCCGACACAGCAAAATGCGTAAGCAAGGTGTGCCGACAGCCCGTGGAAGGTTTGGATTGCGGCGATAGGGTTGCCAGTGGTTAAGCGAAAACCTCGGCATGGAGGGCCTCCGTTTGCTTCGACAATCGG

CSDMCS (+2403) **GCCAGAGAACTCTTCCAAGGATCAGCAGAAGCTAAGTCTGGTTAATCAGGCCAGTTCCTTGCTGCTAAACAAGTCTTCCGTGCGATCGCTTCAATTTGAGGAGCCCCTCGACGAGACTGTGGATCGTTTTTCGGGCAAAATATC**

AC011758 (+2403) GCCAGAGAACTCTTCCAAGGATCAGCAGAAGCTAAGTCTGGTTAATCAGGCCAGTTCCTTGCTGCTAAACAAGTCTTCCGTGCGATCGCTTCAATTTGAGGAGCCCCTCGACGAGACTGTGGATCGTTTTTCGGGCAAAATATC

AF162881 (+2403) GCCAGAGAACTCTTCCAAGGATCAGCAGAAGCTAAGTCTGGTTAATCAGGCCAGTTCCTTGCTGCTAAACAAGTCTTCCGTGCGATCGCTTCAATTTGAGGAGCCCCTCGACGAGACTGTGGATCGTTTTTCGGGCAAAATATC

CSDMCS (+2547) **ATCATCGACACGGGCAGTGCTTTTGAGGAGCTTACCTACAAGGCCCTGTCCATTGGGGGAATCCAATTTTCAGGTGGAGGGTCCCTGCCAGCGCTGCGACATGATCTGCATTAAACCAAGCACAGGCGAACGGTCCGCAGAAAC**

AC011758 (+2547) ATCATCGACACGGGCAGTGCTTTTGAGGAGCTTACCTACAAGGCCCTGTCCATTGGGGGAATCCAATTTTCAGGTGGAGGGTCCCTGCCAGCGCTGCGACATGATCTGCATTAAACCAAGCACAGGCGAACGGTCCGCAGAAAC

AF162881 (+2547) ATCATCGACACGGGCAGTGCTTTTGAGGAGCTTACCTACAAGGCCCTGTCCATTGGGGGAATCCAATTTTCAGGTGGAGGGTCCCTGCCAGCGCTGCGACATGATCTGCATTAAACCAAGCACAGGCGAACGGTCCGCAGAAAC

CSDMCS (+2691) **CTTGACCACCATATCCCGCCTGCAGAAAGGTCGCATGCGATTTCGAGCATCTACATCACTAGGATCCCCAGGACACAAAAGAACTGGAACCCAAGGAGCAACACATGACCTGCGGCGATGTTGTCTTGTGGAATAAGCTCACT**

AC011758 (+2691) CTTGACCACCATATCCCGCCTGCAGAAAGGTCGCATGCGATTTCGAGCATCTACATCACTAGGATCCCCAGGACACAAAAGAACTGGAACCCAAGGAGCAACACATGACCTGCGGCGATGTTGTCTTGTGGAATAAGCTCACT

AF162881 (+2691) CTTGACCACCATATCCCGCCTGCAGAAAGGTCGCATGCGATTTCGAGCATCTACATCACTAGGATCC-----

CSDMCS (+2835) **TTACATCATTAGCGTGGTTTTCTTCGTTCTTTGGTTCGTTGACCCGAAATGTTTTATTGTACATAGTTTTAGCACAAATATCCACTAAGTAATCTCTATTACGTTGCATCAGTTTCGCACTTAGTTTTGTTTTTAAGTAC**

AC011758 (+2835) TTACATCATTAGCGTGGTTTTCTTCGTTCTTTGGTTCGTTGACCCGAAATGTTTTATTGTACATAGTTTTAGCACAAATATCCACTAAGTAATCTCTATTACGTTGCATCAGTTTCGCACTTAGTTTTGTTTTTAAGTAC

CSDMCS (+2979) **TTCTGAAATAGGAATACGCAAAACATTCTTAAAGAAACATTAGGATAGCTTAAGTTCAAAGTAACCGGATACCTCTTGCCAGTGTCTTAAGCCTTTAAAGGCCTTAATTGCAAAATAAACACATTGAAATCGatgtatat**

AC011758 (+2979) TTCTGAAATAGGAATACGCAAAACATTCTTAAAGAAACATTAGGATAGCTTAAGTTCAAAGTAACCGGATACCTCTTGCCAGTGTCTTAAGCCTTTAAAGGCCTTAATTGCAAAATAAACACATTGAAATCGatgtatat

CSDMCS (+3123) **acttgagttccttaggaagggtaatcgaacgtaataaacgcaacgcgtaataaaatgccgatttgaatccaggcgccatttgaagaagtgcgagcagggtaccacgaatcatggaagcaaattagcacaaagttgctt**

AC011758 (+3123) acttgagttccttaggaagggtaatcgaacgtaataaacgcaacgcgtaataaaatgccgatttgaatccaggcgccatttgaagaagtgcgagcagggtaccacgaatcatggaagcaaattagcacaaagttgctt

CSDMCS (+3267) **aacatcaataatgataataatgaataaaaagcatcgcgatatcataacttcatatcacacatatcataaaaattaatgattatatcttcttgaaactgttaaagttatataattaccaaggcttgaaagtggtgagggagt**

AC011758 (+3267) aacatcaataatgataataatgaataaaaagcatcgcgatatcataacttcatatcacacatatcataaaaattaatgattatatcttcttgaaactgttaaagttatataattaccaaggcttgaaagtggtgagggagt

CSDMCS (+3411) **ttgtatcaataactccaaaaatggcatggaagcattttctttcattcattgatttaggacatccttctatctttaaagctgtacggattaattggtacottgttttttgggcttaggtagagcttttatggtctatttatttgg**

AC011758 (+3411) ttgtatcaataactccaaaaatggcatggaagcattttctttcattcattgatttaggacatccttctatctttaaagctgtacggattaattggtacottgttttttgggcttaggtagagcttttatggtctatttatttgg

CSDMCS (+3463) **atataaagcaaacctaagattgaaaactgcagcaaatataactgttgagacca**

AC011758 (+3463) atataaagcaaacctaagattgaaaactgcagcaaatataactgttgagacca

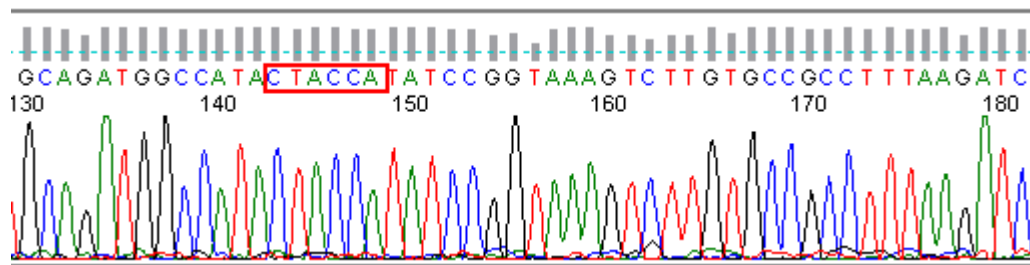
3.6.3. Identification of the mutations responsible for molybdenum cofactor sulfurase deficiencies in *Drosophila melanogaster*.

Once the wild type MCS sequence was confirmed, DNA was extracted from both the *mal-1* and *mal-f1* strains. The MCS region was amplified in both strains and the PCR products were sequenced and compared to the sequence data obtained from the wild type strain.

3.6.3.1. Identification of the mutations responsible for the MFE-deficiency observed in the *mal-1* *Drosophila* strain.

Figure 66 shows a region of the sequence data obtained from primer set DMCS-8 compared to the same region of the wild type sequence.

mal-1:



Wild type:

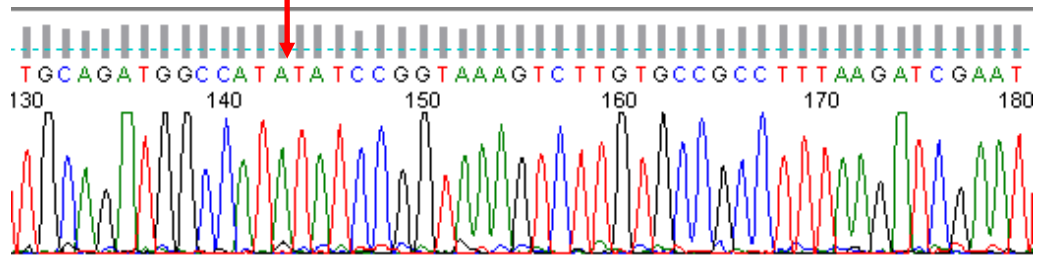


Figure 66: Comparison of the DNA sequencing electrophoretograms obtained from the wild type and the *mal-1* *D. melanogaster* strains using primer set DMCS-8.

Top electrophoretogram shows sequence obtained from *mal-1* strain. Bottom electrophoretogram shows sequence obtained from wild type strain. The red box highlights a 6 bp insertion in *mal-1* and the red arrow indicates the point of insertion in wild type.

Comparison of the sequence data obtained from the *mal-1* strain revealed a 6 bp insertion within exon 4 of the MCS gene (Figure 67). The mutation was identified at nucleotide position c.2123_2124insCTACCA from the AUG initiator codon in the predicted mRNA transcript of the *mal-1* mutant. Translation of the predicted mRNA indicated that the 6 bp insertion resulted in the in-frame p.Ile577_Tyr578insLeuPro (Figure 67).

(a)

```
DMCS/mal-1 (+2094) TTGAGGCCAAAACCTATTGCAGATGGCCATACTACCATATCCGGTAAAGTCTTGTGCCGCC
CSDMCS (+2094) TTGAGGCCAAAACCTATTGCAGATGGCCATA-----ATATCCGGTAAAGTCTTGTGCCGCC
*****
```

(b)

```
CSDMCS (540) ATKPLQRIQFIEEQAEQLPPLLKERVQLLRPKLLQMAI--YPVKSCAAFKIELPGSWPLT
DMCS/mal-1 (540) ATKPLQRIQFIEEQAEQLPPLLKERVQLLRPKLLQMAILPYPVKSCAAFKIELPGSWPLT
*****
```

Figure 67: Comparison of the *mal-1* with Canton-S wild type molybdenum cofactor sulfurase nucleotide and deduced protein sequences at the site of the mutation.

(a) Nucleotide sequence alignment of the Canton-S and *mal-1 Drosophila* a mutant strain, which was obtained when the PCR product was aligned using CLUSTAL X program (Larkin *et al.*, 2007). The 6bp insertion is shown in red text. (b): Predicted protein sequence of the *mal-1* mutant. In frame addition of two amino acids are resulting from the 6bp insertion shown in red text were aligned using CLUSTAL X program (Larkin *et al.*, 2007). CSDMCS = Canton-S molybdenum cofactor sulfurase. DMCS/*mal-1* = molybdenum cofactor sulfurase of *mal-1* strain

3.6.3.2. Identification of the mutations responsible for the MFE-deficiency observed in the *mal-f1* *Drosophila* strain.

When the sequence data obtained from the *mal-f1* strain was compared to that of the wild type strain a mutation was identified in exon 4 of the MCS gene (Figure 68).

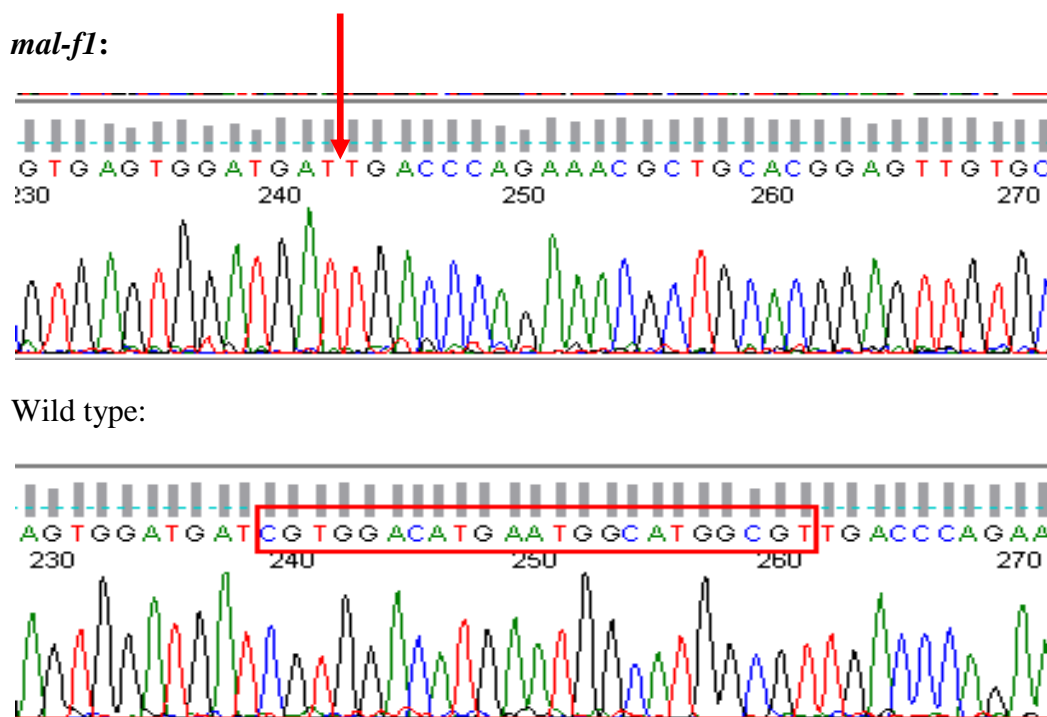


Figure 68: Comparison of the sequencing electrophoretograms of the MCS gene from the wild type and the *mal-f1* *D.melanogaster* strains using primer set DMCS-8.

Top electrophoretogram shows sequence obtained from *mal-f1* strain and the red arrow indicates the points of deletion in *mal-f1*. Bottom electrophoretogram shows sequence obtained from wild type strain. Red box highlights a 23 bp deletion.

Comparison of the sequence data obtained from the *mal-f1* and wild type strains identified a 23bp deletion c.2219_2220delCGTGGACATGAATGGCATGGCGT (Figure 69).

```

DMCS-mal-f1 (+2173) GGCCATTGACGGATCAAGGTTTGAAATATGACCGTGAGTGGATGAT-----
CSDMCS (+2173) GGCCATTGACGGATCAAGGTTTGAAATATGACCGTGAGTGGATGATCGTGGACATGAATG
*****
DMCS-mal-f1 (+2233) -----TGACCCAGAAACGCTGCACGGAGTTGTGCCTAATTAGGCCAGTGATTAAGG
CSDMCS (+2233) GAATGGCGTTGACCCAGAAACGCTGCACGGAGTTGTGCCTAATTAGGCCAGTGATTAAGG
*****
DMCS-mal-f1 (+2293) TTGACCAATTGGAGTGCAGTTTGGAGAAAATCCACCATCTCGGTGCCCTTTCTCTGGA
CSDMCS (+2293) TTGACCAATTGGAGTGCAGTTTGGAGAAAATCCACCATCTCGGTGCCCTTTCTCTGGA
*****

```

Figure 69: Nucleotide sequence alignment of the Canton-S and *mal-f1* molybdenum cofactor sulfurase gene at the site of the 23bp deletion in exon 4.

The 23bp deletion is shown in red text. CSDMCS = Canton-S molybdenum cofactor sulfurase. DMCS/*mal-1* = molybdenum cofactor sulfurase of *mal-f1* strain.

Translation of the predicted mRNA from the *mal-f1* strain identified that the 23 bp deletion resulted in a frame shift at codon position 550 and the addition of a premature stop codon at position 562 (Figure 70).

```

CSDMCS      MTSYRPEFSASEQSQIDAEFSRLASNKSVYLDHAGTTLYAESQVTAAAEQLQRNVICNPH (60)
DMCSmal-f1  MTSYRPEFSASEQSQIDAEFSRLASNKSVYLDHAGTTLYAESQVTAAAEQLQRNVICNPH (60)
*****
CSDMCS      TCRLTGDFVDQVRFKILEFFNTTAEDYHVIPTANATAALSLVAENFDGSSGEFHFQEN (120)
DMCSmal-f1  TCRLTGDFVDQVRFKILEFFNTTAEDYHVIPTANATAALSLVAENFDGSSGEFHFQEN (120)
*****
CSDMCS      HTSVLGMREVRRENGIYMLRENEISGGKHKANGKVHEVSGKTGNSLLTFSACNFSGYKI (180)
DMCSmal-f1  HTSVLGMREVRRENGIYMLRENEISGGKHKANGKVHEVSGKTGNSLLTFSACNFSGYKI (180)
*****
CSDMCS      PLEVIEQIQIDGLAKPGKELWSSLGEKKNMHNDYYICLDAASFVATSPDLQKYRPDYV (240)
DMCSmal-f1  PLEVIEQIQIDGLAKPGKELWSSLGEKKNMHNDYYICLDAASFVATSPDLQKYRPDYV (240)
*****
CSDMCS      CLSFYKIFGYPTGVGALLVSRGAEVFKRRFFGGGTINYAYPHAMDYQLRETFHQRYED (300)
DMCSmal-f1  CLSFYKIFGYPTGVGALLVSRGAEVFKRRFFGGGTINYAYPHAMDYQLRETFHQRYED (300)
*****
CSDMCS      GTLPFLSIVGLLEGFRTLRLVPRTEDEFSTMERISRHVFLAKYLEDQLRQLHHPNGEPL (360)
DMCSmal-f1  GTLPFLSIVGLLEGFRTLRLVPRTEDEFSTMERISRHVFLAKYLEDQLRQLHHPNGEPL (360)
*****
CSDMCS      VKLYNKVGYQDKSRQGGIVAFNVRTESGSFVGFGEIACVAALHGILLRTGCFNIGACQY (420)
DMCSmal-f1  VKLYNKVGYQDKSRQGGIVAFNVRTESGSFVGFGEIACVAALHGILLRTGCFNIGACQY (420)
*****
CSDMCS      YLGLDEDALDAIYKRAGRICGDFDLIDGQPTGAVRVSFYGMTTIQDQVQLQLMRSSYL (480)
DMCSmal-f1  YLGLDEDALDAIYKRAGRICGDFDLIDGQPTGAVRVSFYGMTTIQDQVQLQLMRSSYL (480)
*****
CSDMCS      ATKPLQRIQFIEEQAEQLPPLLKERVQLLRPKLLQMAIYPVKSCAAFKIELPGSWPLTDQ (540)
DMCSmal-f1  ATKPLQRIQFIEEQAEQLPPLLKERVQLLRPKLLQMAIYPVKSCAAFKIELPGSWPLTDQ (540)
*****
CSDMCS      GLKYDREWMIVDMNGMALAQKRCTELCLIRPVIKVDQLELQFGENSTISVPLSLDDQAAD (600)
DMCSmal-f1  GLKYDREWMIDPETLHGVVFN* (561)
*****
CSDMCS      TAKCVSKVCRQPVVEGLDCGRVAQWLSENLMGELRLRLRQSGQRNSSKDQQKLSLVNQAQ (660)
CSDMCS      FLLLNKSSVRSLOFEEPLDETVDVDFRANIIIDTGSAFEELTYKALSIGGIQFQVEGPCQR (720)
CSDMCS      CDMICINQRTGERSPETLTITISRLQKGRMRFGIYITRIPQDTKELEPKQHMTCGDVVLV (780)
CSDMCS      E (781)

```

Figure 70: Comparison of the predicted molybdenum cofactor sulfurase protein sequence of the *mal-f1* and Canton-S wild type strain. Sequences were aligned using CLUSTAL X program (Larkin *et al.*, 2007).

Red text indicates the altered amino acid sequence at the carboxyl-terminus in the *mal-f1* mutant strain. CSDMCS = Canton-S molybdenum cofactor sulfurase. DMCS/*mal-1* = molybdenum cofactor sulfurase of *mal-f1* strain.

4. Discussion.

This discussion section follows the order of the results section.

4.1.1. Spectrophotometric and HPLC investigation of molybdoflavoenzymes characterisation in wild type Canton-S *Drosophila* strain.

Only two studies have directly measured the kinetics of substrates disappearance or product generation on *Drosophila melanogaster* AO. Wurzinger and Hartenstein (1974) used vanillin as substrate of *Drosophila* AO with O₂ as an electron acceptor in a spectrophotometric assay (Wurzinger & Hartenstein, 1974) and a fluorometric assay was used for acetaldehyde as substrate of AO (Dickinson & Gaughan, 1981). Rather than directly measure substrate disappearance or product generation AO activity many studies measure AO activity indirectly using artificial electron acceptors. Several studies have used acetaldehyde substrate with 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor for aldehyde oxidase in a spectrophotometric assay (Browder & Williamson, 1976; Dickinson, 1971; Dickinson & Gaughan, 1981; Wahl *et al.*, 1982; Warner & Finnerty, 1981) and heptaldehyde was used as a substrate with DCPIP as an artificial electron acceptor (Cypher *et al.*, 1982). The majority of previous studies on *Drosophila* AO have been using a semi-quantitative histochemical assay for acetaldehyde, benzaldehyde, anisaldehyde, *p*-chlorobenzaldehyde, cinnamaldehyde, citral, citronellal, dimethylaminobenzaldehyde, hexaldehyde, *p*-hydroxybenzaldehyde, *m*-hydroxybenzaldehyde, *o*-hydroxybenzaldehyde, salicylaldehyde, pyridoxal and vanillin substrates of *Drosophila* AO with phenazine methosulfate as an artificial electron acceptor (Cypher *et al.*, 1982; Dickinson & Gaughan, 1981). Interestingly, no AO N-heterocyclic substrates or retinaldehyde that are a major important class of substrates for mammalian aldehyde oxidase (Table 1) (Garattini *et al.*, 2008; Kitamura *et al.*, 2006) have been investigated as substrates for

Drosophila AO. In this study N-heterocyclic compounds including drugs and the vitamin metabolite (N¹-methylnicotinamide) and vitamin A (retinaldehyde) were used in the first time for *Drosophila* AO using sensitive spectrophotometric and HPLC methods as a new direct assay for determining the AO activity for *Drosophila melanogaster*. In the AO spectrophotometric assays one N-heterocyclic compound, phenanthridine was used as a substrate. Two aldehyde compounds, vanillin and dimethylaminocinnamaldehyde, were also used as aldehyde substrates of AO. XDH was also studied using its endogenous substrate, xanthine in a spectrophotometric and HPLC assay.

Prior to testing with the large number of substrates the optimal conditions of the activities of both molybdo-flavoenzymes were studied in *Drosophila*. The activity of AO and XDH of *Drosophila* was measured as at different incubation temperatures for 30 minutes as described in section 2.8.2.1. The rate of reaction of *Drosophila* AO with DMAC gradually rose with increase temperature from 20°C up to 37°C, then the activity decreased above this value (Figure 29). A similar result was obtained with *Drosophila* XDH where the activity of XDH enzyme rose with increasing temperature up to 45°C, and these results resemble those with most animal enzymes that become denatured at temperatures above 40°C (Figure 29) (Pfeiffer, 1954). This present result showed that the assay temperature of 37°C is suitable for use with the *Drosophila* MFE enzymes.

The effect of pH on *Drosophila* AO and XDH was assayed under standard assay condition, except that the reaction pH was varied between 5.6 and 8.0 as described in section 2.8.1. The maximum activity of AO and XDH was achieved at a similar pH optimum between pH 7 and 7.5 as shown figure 30. These result agree with Egwim *et al.* 2005 studied which found that the optimum pH of cow and goat milk XOR were

7.5 (Egwim *et al.*, 2005). The optimum pH for AO from *Drosophila melanogaster* was observed in the present study is similar to that found by Stoddart *et al.* who found the optimal pH of rabbit AO was 7.5, when oxygen was used as the electron acceptor (Stoddart & Levine, 1992).

K_m and V_{max} were determined from the Eadie- Hofstee plot of V versus $V/[S]$ as described in the section materials and methods using simple rapid spectrophotometric assays. Enzyme activity of AO was measured spectrophotometrically using three different substrates with O_2 as electron acceptor as follows. These were chosen for the following reasons. The DMAC is a specific substrate for rat AO (Kurth & Kubiciel, 1984; Maia & Mira, 2002; Moriwaki *et al.*, 1998) and phenanthridine has been shown to be a good specific substrate for monitoring mammalian AO activity in many studies (Beedham *et al.*, 1989; Lake *et al.*, 2002; Pirouzpanah *et al.*, 2006; Sorouraddin *et al.*, 2008; Terao *et al.*, 2000). Vanillin has also been shown to be a good substrate with a high affinity for AO guinea pig liver (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004).

The lowest K_m value for an AO substrate was found with DMAC being $50 \pm 5.4 \mu M$ (mean \pm SD) with Canton-S cytosol. In agreement with the present result Li *et al.* (2009) found that the DMAC is an excellent substrate for rat liver AO with a low K_m value ($9.6 \mu M$) (Li *et al.*, 2009). The results of this study indicated that DMAC is better substrate of *Drosophila* AO than the phenanthridine ($K_m = 80 \pm 9.1 \mu M$) and vanillin ($K_m = 303 \pm 11.7 \mu M$). The K_m value for *Drosophila* AO with vanillin was approximately 2.5 times greater than that found guinea pig liver AO that has a K_m value of $120 \mu M$ (Panoutsopoulos *et al.*, 2005). In contrast, the *Drosophila* enzyme has a 100 fold difference in its K_m value for vanillin than the human enzyme. Obach, (2004) found vanillin is an excellent substrate for human liver AO with a low K_m (2.7

μM) (Obach, 2004). Cypher *et al.* (1981) found the K_m value for heptaldehyde as substrate for *Drosophila* AO was 2500 μM . This suggests that the aromatic aldehyde vanillin is a better substrate for *Drosophila* AO than the aliphatic aldehyde heptaldehyde. Phenanthridine as a specific substrate of AO has been used in several papers and K_m previously has been estimated were $< 1 \mu\text{M}$ with rabbit and guinea pig liver AO enzyme and 6 μM and 14 μM with rat and human liver AO, respectively (Beedham, 2001; Rashidi *et al.*, 1997; Stubley & Stell, 1980). The present result is indicated that phenanthridine a good substrate of *Drosophila* AO, in terms of a K_m of phenanthridine ($K_m = 80 \pm 9.1 \mu\text{M}$) (mean \pm SD) but this value is considerably greater than the previously studies of mammalian AO. Comparison of the K_m values between the different *Drosophila* strains showed that there were no significant differences between Canton-S and ry^{506} strains for phenanthridine and DMAC as substrates. However, the K_m of vanillin obtained for the ry^{506} strain was significant lower than that with Canton-S. This might be due to a polymorphism in the *Drosophila* AO gene. Further time consuming and costly molecular genetic studies would be required to clarify this however.

Kinetic analyses of XDH enzymes revealed that xanthine is the preferred substrate for XDH (Self & Stadtman, 2000). The K_m for XDH with xanthine using NAD^+ as an electron acceptor was $27 \pm 4.1 \mu\text{M}$ (mean \pm SD) with Canton-S cytosol (Table 23). These results supported by the finding of Edwards *et al.* (1977) and Doyle *et al.* (1996) results, who found that K_m of the Canton-S wild type of *Drosophila* was 29 μM and 24 μM respectively in xanthine hydroxylation assay (Doyle *et al.*, 1996; Edwards *et al.*, 1977). Krenitsky *et al.* (1986) reported that the xanthine is an excellent substrate of liver human XO and bovine milk XO with a low K_m (7 μM and 8 μM ,

respectively) (Krenitsky *et al.*, 1986). Thus the *Drosophila* enzyme is has an approximately 4-fold weaker affinity for xanthine than its mammalian ortholog.

4.1.2. Spectrophotometric and HPLC investigation of molybdoflavoenzyme characterisation in transgenic and molybdoflavoenzyme deficient *Drosophila* strains.

Following the assay optimisation and kinetic measurements with wild type Canton-S, spectrophotometric assays were also made using DMAC, phenanthridine, vanillin and xanthine in transgenic, *ry*⁵⁰⁶ and maroon-like strains. XDH activity in *ry*⁵⁰⁶ and maroon-like strains was absent (Table 23 using the spectrophotometric assay) and this was confirmed by a sensitive HPLC assay with extended assay times (Figure 32). It was therefore confirmed that the *ry*⁵⁰⁶ and maroon-like strains were deficient in XDH that they were *bona fide* contamination-free stocks that could be used in this research study. Spectrophotometric and HPLC assays of vanillin incubated with fly extracts of transgenic and *ry*⁵⁰⁶ strains showed the conversion of vanillin to vanillic acid by AO (Figure 31). These results confirmed that the key enzyme responsible for the oxidation of vanillin to vanillic acid is AO as the *ry*⁵⁰⁶ strain is XDH deficient. Canton-S, transgenic and *ry*⁵⁰⁶ had similar activities but the *ry*⁵⁰⁶ was slightly higher than in Canton-S. This might have been due to a compensation effect due to the fact that *ry*⁵⁰⁶ strain has only got AO and Canton-S has both AO and XDH. It is hypothesised that the higher AO activity present in *ry*⁵⁰⁶ mutants may be to compensate for not having XDH, possibly due to up regulation of AO gene transcription. This results agree with Panoutsopoulos *et al.* (2004) who indicated that the guinea pig liver AO is able to convert vanillin to vanillic and that vanillin is not a good substrate for milk xanthine oxidase (Panoutsopoulos *et al.*, 2004). When the transgenic and *ry*⁵⁰⁶ strains cytosol were incubated with phenanthridine as substrate 6(5H)-phenanthridone was produced (Figure 31). This result agrees with the several papers reported that the phenanthridine

is a good substrate and has a very high affinity towards AO (Beedham, 2001; Rashidi, 1996; Stubley & Stell, 1980). In order to determine that there was definitely no activity present in maroon-like strains for phenanthridine and vanillin a HPLC assay was used to confirm lack of product formation (Figure 31).

Table 22 shows a summary of the all AO assay results in *ry*⁵⁰⁶ and *ma-l* strains. No DMAC, phenanthridine and vanillin oxidase activity was found in *ma-l* strains, but *ry*⁵⁰⁶ strain had AO activity for these substrates. This confirmed that AO enzyme is deficient in the *ma-l* strains. These results are consistent with Browder and Williamson (1976) who found the AO activity was completely missing in *ma-l* strain when acetaldehyde was used as a substrate and DCPIP was the electron acceptor (Browder & Williamson, 1976).

4.1.3. *In vitro* Quantification of molybdo-flavoenzyme activities in wild type, transgenic and mutant *Drosophila melanogaster* strains by HPLC assays.

A high performance liquid chromatography with UV and diode array detector was used for the determination of the conversion of AO and XOR substrates and products that did not have simple spectrophotometric assays. For oxidation reactions, substrates in this study have been classified into two structure types: aldehydes, which undergo oxidation to carboxylic acids and aromatic azaheterocyclic compounds, which undergo oxidation to lactams.

All HPLC protocols for analytes were tested for method linearity, accuracy and precision were checked. Regression for calibration (r^2) standards injected were more than 0.997 for all analytes tested. Accuracy for the method was more than 95% and precision < 3.5%, calculated by using three injections on the same day as calibration standards (intraday) (Tables 2 and 3 in appendix 3). For the linearity for the results,

the lower limit of Quantification (LOQ) and limit of detection (LOD) were determined for each analyte (Table 4 in appendix 3). According to (Miller & Miller, 2005) these methods have acceptable limits for both accuracy and precision and were therefore used to analyze samples from this study.

4.1.3.1. Biotransformation of xanthine and methylxanthines by *D. melanogaster* strains

The HPLC chromatogram in figure 35 represents incubated Canton-S cytosol (wild type) with xanthine in which NAD^+ was present. The uric acid peak was only produced with NAD^+ as electron acceptor (Figure 35). No uric acid was produced when O_2 was the sole electron acceptor. These finding agree with Hughes *et al.* (1992) who observed that XDH of wild type *Drosophila* strains converted xanthine to uric acid only with NAD^+ present (Hughes *et al.*, 1992). The XOR inhibitor oxipurinol was used to investigate that the uric acid came from oxidation of xanthine by XDH (Figure 36). The chromatogram produced from the incubation of Canton-S cytosol, xanthine, NAD^+ and oxipurinol that showed the peak of uric acid decreased dramatically to approximately 90% with enzyme inhibitor present. This agrees with Wahl *et al.* (1982) who reported that allopurinol is a potent inhibitor of *Drosophila* XOR with pterin as a substrate (Wahl *et al.*, 1982). Canton-S cytosol was also able to convert 1-methylxanthine to 1-methyluric acid when NAD^+ was present as an electron acceptor but no product was formed without NAD^+ (Figure 36). In contrast the mono-methylxanthines of 3 and 7-methylxanthine were not converted to their uric acid metabolites by *Drosophila* cytosol. This suggests that the position of the methyl group in mono-methylated xanthines has a dramatic effect on the oxidation of methylxanthines substrates in *Drosophila*. These results agree with Reinke *et al.* (1987) who found the XDH of rat can catalyse the oxidation of 1-methylxanthine to 1-

methyluric acid, but could not metabolise 3- methylxanthine and 7- methylxanthine to 3- methyluric acid and 7-methyluric acid respectively (Reinke *et al.*, 1987). Krenitsky *et al.* also reported that the catalysis of mono-methylated xanthine by bovine milk XOR was affected by the position at which oxidation occurred in purine ring. The bovine milk xanthine oxidase catalyses the oxidation of 7-methylxanthine, 1-methylxanthine and theophylline, to varying degrees with the 1-methylxanthine being the best substrate (Krenitsky *et al.*, 1972). HPLC analysis of XDH activity in rosy and maroon-like strains revealed a lack of any detectable uric acid, 1, 3 and 7 methyluric acid peaks, even after extended incubation times of 120 minutes. These results are consistent with Wahl *et al.*, (1982) who did not detected XDH activity in *ma-l* and *ry*⁵⁰⁶ strains respectively.

4.1.3.2. Biotransformation of 6-mercaptopurine and methotrexate by *D. melanogaster* strains

In the present study, 6-MP was incubated with cytosolic fraction of all *Drosophila* strains. According to the *in vitro* studies, 6-MP is not a substrate for both XOR and AO of *Drosophila melanogaster* as no metabolites were detected with or without NAD⁺ in the incubation (Figure 37). In contrast when rabbit liver cytosol was incubated with 6-MP the 6-MP was oxidized to 6-thiouric acid via 6-thioxanthine (Figure 38). This is in agreement with Hall and Krenitsky (1986) who showed that, *in vitro* 6-MP is a substrate for rabbit liver AO with a relatively low K_m of 1.6 mM (Hall & Krenitsky, 1986) but is poor a substrate for bovine milk xanthine oxidase with a low oxidation rate (Krenitsky *et al.*, 1972). In contrast, Rashidi *et al.* (2007) reported that in the *in vitro* biotransformation of 6-MP with guinea pig liver fractions, XO enzyme had a major contribution to the oxidation of 6-MP to 6-thioxanthine, while the

XO and AO enzyme both catalysed the oxidation the 6-thioxanthine to 6-thiouric acid via 6-thioxanthine (Rashidi *et al.*, 2007).

The anti-folate agent methotrexate (MTX) is widely used in the treatment of acute lymphocytic leukemia in children (Kitamura *et al.*, 1999). MTX was mainly oxidized to 7-hydroxymethotrexate (7-OH-MTX) by AO but has widely different transformation rates in different species (Beedham, 1985; Kitamura *et al.*, 1999; Kuroda *et al.*, 2000; Moriyasu *et al.*, 2006; Yu *et al.*, 1989). Even after a 4 hrs incubation the MTX was not converted to its metabolite with any *Drosophila* strains (Figure 44). Because of the results showed no activity of *Drosophila* AO with MTX, in order to confirm that the experimental conditions were correct MTX was incubated with rabbit liver cytosol using the same incubation conditions as for *Drosophila* samples (Figure 44). 7-OH-MTX was formed by the action of AO from the rabbit liver cytosol, due to the rabbit liver AO having a very high hydroxylating activity towards this substrate compared with other species (Johnson *et al.*, 1966; Jordan *et al.*, 1999; Kitamura *et al.*, 1999). The lack of MTX-hydroxylation activity in *Drosophila* is not that surprising as MTX oxidase activity varies greatly between species. MTX oxidase activity is highest in rabbits followed by rats, hamsters and monkeys but is undetectable in dogs (Kitamura *et al.*, 1999). *In-vitro* human liver AO catalyses the oxidation of MTX to 7-OH-MTX at a relatively low rate (Jordan *et al.*, 1999; Kitamura *et al.*, 1999).

4.1.3.3. Biotransformation of allopurinol by *D. melanogaster* strains

Allopurinol is a prodrug that is metabolised to oxipurinol by the action of both AO and XOR (Ichida *et al.*, 1998; Pacher *et al.*, 2006; Reiter *et al.*, 1990; Yu *et al.*, 2009). It was used as a substrate for determining if *ry*⁵⁰⁶ and *ma-l* strains were good animal models for hereditary xanthinuria type I and II respectively. Using O₂ as an electron

acceptor Canton-S strain biotransformed allopurinol to oxipurinol is suggesting AO involvement in this catalysis (Figure 39). The addition of NAD^+ further enhanced the conversion, indicating the involvement of XDH as is the situation in normal humans. To confirm the hypothesis that AO plays a role in the oxidation of allopurinol to its metabolite, the ry^{506} cytosol that is XOR deficient was incubated with allopurinol. Mimicking the findings with human hereditary xanthinuria type I, the chromatogram (Figure 40) showed that ry^{506} strain can metabolise allopurinol to oxipurinol without the XDH enzyme indicating that like human AO (Reiter *et al.*, 1990; Yamamoto *et al.*, 1991) and rat AO (Moriwaki *et al.*, 1993) the *Drosophila* AO also contributes to the oxidation of allopurinol as in type I hereditary xanthinuria. In comparison, the results obtained from this *in vitro* study showed that the *mal-1* and *mal-fl* strains of *Drosophila melanogaster* that are deficient in both AO and XDH were not able to generate oxipurinol following incubation with allopurinol even with extended incubation times. This is similar to the situation reported by Yamamoto *et al.* (1989), Reiter *et al.* (1990), Yamamoto *et al.* (1991) and Simmonds, (2003) who found that patients with hereditary xanthinuria type II cannot metabolise allopurinol to oxipurinol. This confirms that the maroon-like mutants that have MCS gene mutations are good animal models for this human dual enzyme genetic deficiency.

4.1.3.4. Biotransformation of pyrazinamide by *D. melanogaster* strains

Pyrazinamide is a drug used to treat tuberculosis that is metabolised to 5-hydroxypyrazinamide by the action of XOR and AO in mammals (Kitamura *et al.*, 2006; Mehmedagic *et al.*, 2002; Mehmedagic *et al.*, 1997; Moriwaki *et al.*, 1997). When pyrazinamide was incubated with Canton-S and ry^{506} cytosol 5-hydroxypyrazinamide was produced (Figures 40 and 41). To confirm if *Drosophila* XDH could also contribute to pyrazinamide hydroxylation, pyrazinamide was

incubated with Canton-S cytosol with and without NAD^+ and HPLC analysis were carried out. After incubation with Canton-S cytosol with NAD^+ the HPLC chromatogram showed a 18% increased amount of 5-hydroxypyrazinamide when compared with the incubation without NAD^+ (Figure 40). This indicated that pyrazinamide is a substrate for both enzymes. This result was consistent with finding in previous *in vitro* study, which confirmed that both AO and XOR can oxidise pyrazinamide to 5-hydroxypyrazinamide in humans. In addition Yamamoto *et al.* (1989) suggested that AO may play a major role in the oxidation of pyrazinamide to 5-hydroxypyrazinamide in the xanthinuria type I patient (Yamamoto *et al.*, 1989). A similar situation to the human xanthinuria type I was found with ry^{506} flies. The production of 5-hydroxypyrazinamide peak by the ry^{506} strain indicates that AO can substitute for XOR in the conversion of pyrazinamide to 5-hydroxypyrazinamide. This result mimics the situation in humans in which patients with xanthinuria type I that have a normal activity of AO were able to convert pyrazinamide to 5-hydroxypyrazinamide at a normal rate (Yamamoto *et al.*, 1989). In experiments in this study there was no conversion of pyrazinamide to 5-hydroxypyrazinamide by maroon-like strains. This proved the *mal-1* and *mal-f1* flies were deficient in both AO and XDH as no pyrazinamide oxidase activity was seen and resembled the situation found in human hereditary xanthinuria type II patients (Yamamoto *et al.*, 1989). Unfortunately there is no available commercial standard of 5-hydroxypyrazinamide to confirm the identity of this metabolite peak and due to the low concentration of this metabolite no results were obtained by LC-MS.

4.1.3.5. Biotransformation of cinchonine by *D. melanogaster* strains

The cinchona alkaloids have been widely used the treatment of malaria (Hunter, 1988). All four cinchona alkaloids, quinine, quinidine, cinchonine and cinchonine, are

oxidised by AO to the 2'-quinolone derivatives in mammals (Al-Tayib, 2009; Beedham *et al.*, 1992; Palmer *et al.*, 1969; Stubley *et al.*, 1979; Zhao & Ishizaki, 1997). With incubation of Canton-S and *ry*⁵⁰⁶ cytosol a metabolite of cinchonine (- cinchoninone) was produced (Figure 43). The *mal-1* and *mal-fl* strains thought to be deficient showed no oxidation of cinchonine even after extended times compared with Canton-S, transgenic and *ry*⁵⁰⁶ strains. These results indicate *Drosophila* AO plays an important role in the metabolism of cinchonine. Unfortunately there is no available commercial standard of 2'-cinchoninone to confirm the identity of this metabolite peak and due to the low concentration of this metabolite no results were obtained by LC-MS.

4.1.3.6. Biotransformation of vitamin B6 (pyridoxal) in *D. melanogaster* strains

Vitamin B6 is a water-soluble compound that contains a pyridine ring. Vitamin B6 is present in nature as several different forms such as pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and their active form is pyridoxal 5'-phosphate (PLP) forms (Fitzpatrick *et al.*, 2007). PLP is the coenzymatically active form of vitamin B6 and plays an important role in maintaining the biochemical homeostasis of the body (Meister, 1990; Snell, 1990). There are more than 100 PLP-dependent enzymes in a cell that perform essential roles in various metabolic pathways including amino acid metabolism (such as amino acid synthesis and degradation), fatty acid metabolism (such as the synthesis of polyunsaturated fatty acids) and carbohydrate metabolism (such as the breakdown of glycogen) (Mooney *et al.*, 2009). The preferred degradation route from PLP to 4-pyridoxic acid involves the dephosphorylation of PLP by the phosphatase (Jang *et al.*, 2003) followed separately by the actions of aldehyde oxidase and β -nicotinamide adenosine dinucleotide-dependent dehydrogenase (Schwartz & Kjeldgaard, 1951; Stanulovic *et al.*, 1976). *Drosophila* has been documented as

having at least two of these degradation enzymes, namely pyridoxal oxidase and PLP-dependent (Jang *et al.*, 2003).

Pyridoxal oxidase was identified in *D. melanogaster* by Forrest *et al.* (1961) and was demonstrated by its ability to catalyse the oxidation of pyridoxal to pyridoxic acid (Forrest *et al.*, 1961). Further studies by Keller and Glassman, (1964) suggested that pyridoxal oxidase was closely related to aldehyde oxidase (Keller & Glassman, 1964) as it had a similar ontogeny and joint regulation (Browder & Williamson, 1976).

When pyridoxal was incubated with Canton-S, transgenic and *ry*⁵⁰⁶ *Drosophila* cytosol pyridoxic acid was produced (Figure 45). No pyridoxic acid was produced by the *ma-l* strains. The result confirms that conversion of pyridoxal to its metabolite due to the AO enzyme as in wild type strain in agreement with Cypher *et al.* (1982) who found that pyridoxal oxidase was present in wild type *Drosophila melanogaster* using a histochemical assay (Cypher *et al.*, 1982). Similarly Warner and Finnerty, (1981) confirmed the activity of pyridoxal oxidase in wild type *Drosophila* and it's absent in *ma-l* strains by measuring indirectly using DCPIP as an artificial electron acceptor in a spectrophotometric assay. The *in vitro* result in this study is reflected in the study by Kamleh *et al.* (2009) that showed *ma-l* strains did not produce pyridoxic acid in contrast to wild type strains (Kamleh *et al.*, 2009).

4.1.3.7. Biotransformation of vitamin A (all-trans-retinaldehyde) in *D. melanogaster* strains

Another potential substrate of AO of physiological importance is all-trans-retinaldehyde which oxidised it to retinoic acid (Huang *et al.*, 1999). Retinoic acid is a key regulator of the homeostasis of keratinized epithelia and a recognized morphogen of the vertebrate organisms (Chambon, 1996) although its role in *Drosophila* is less well understood. Retinal congeners are components of rhodopsin

visual system in all vertebrate and invertebrates including *Drosophila* (Lee *et al.*, 1996; Seki *et al.*, 1998). Interestingly the retinal determination network that is responsible for controlling eye development is one of the most extensively studied gene regulatory networks in both invertebrate and invertebrate species (Datta *et al.*, 2011). *Drosophila* mutants have been the most important organism in delineating this network (Datta *et al.*, 2011). Although the genes and proteins that control eye development are fully understood proteins that might regulate the levels of retinal and retinoic acid in *Drosophila* are poorly understood. To date however there has been retinal oxidase documented activity in *Drosophila*.

In this study, it was important to establish if *Drosophila* AO is capable of oxidizing retinaldehyde to its metabolite retinoic acid like vertebrate AO (Garattini *et al.*, 2008; Garattini & Terao, 2011, 2012; Huang *et al.*, 1999; Huang & Ichikawa, 1994; Kitamura *et al.*, 2006; Terao *et al.*, 2009). On incubation of Canton-S, transgenic and *ry*⁵⁰⁶ *Drosophila* cytosol with all-*trans*-retinaldehyde this generated retinoic acid (Figure 45). These finding are consistent with Huang and Ichikawa, (1994) and Tsujita *et al.* (1994) who first observed the role of AO in the oxidation of all-*trans*-retinaldehyde to retinoic acid without NAD⁺ in rabbit by liver AO cytosol (Huang & Ichikawa, 1994; Tsujita *et al.*, 1994). The maroon-like strains (*mal-1* and *mal-f1*), which lack AO were not able to catalyse the oxidation all-*trans*-retinal confirming that AO in *Drosophila* is responsible for this conversion.

In mice a knockout model that is deficient in retinal oxidase activity although viable and fertile has perturbations and cellular secretion. In addition this model also had up regulation of genes involved in hypertrophic responses upon UV exposure (Terao *et al.*, 2009). In view of a lack of retinal oxidase activity, reduced lifespan and reduced tolerance to chemical and physical stresses it in maroon-like mutants it would be

interesting to use transcriptomic and proteomic analysis to determine if similar pathological responses were present in this strain of *Drosophila* as the ones found in mice.

4.1.3.7. Biotransformation of N¹-methylnicotinamide by *D. melanogaster* strains

N¹-methylnicotinamide (NMN) is often used as a marker for aldehyde oxidase (Beedham, 1987; Felsted & Chaykin, 1967; Stanulovic & Chaykin, 1971; Sugihara *et al.*, 2006). NMN which is formed from nicotinamide by nicotinamide N-methyltransferase which is widely distributed in animals (Yan *et al.*, 1997). Many findings are consistent with the role of AO in the conversion of NMN to 2-PY and 4-PY in the final steps of the nicotinamide degradation pathway (Beedham, 1987; Felsted & Chaykin, 1967; Hava Peretz *et al.*, 2011; Stanulovic & Chaykin, 1971; Sugihara *et al.*, 2006). As *Drosophila* utilises NAD⁺ as a cofactor for multitudes of enzymatic reactions by dehydrogenases it is reasonable to suggest that *Drosophila* may also have pathways for nicotinamide degradation and have NMN oxidase activity however to date there is no report of this.

When NMN was incubated with Canton-S, transgenic and *ry*⁵⁰⁶ cytosol two product peaks were found (Figure 46) suggesting that there was AO activity towards this compound. Based on what is found in mammals it is assumed that these might be N¹-methyl-2-pyridone-5-carboxamide (2-PY) and N¹-methyl-4-pyridone-5-carboxamide (4-PY) (Figure 46). The metabolism of NMN to 2-PY and 4-PY has been reported to be catalysed by AO in a number of mammals including humans (Felsted & Chaykin, 1967; Stanulovic & Chaykin, 1971; Sugihara *et al.*, 2006). NMN showed different metabolic profiles in mice and humans by AO *in vivo* and *in vitro*; for example the major product was 2-PY in humans, whereas in mice were 2-PY and 4-PY (Kitamura *et al.*, 2008). The ratio of 2-PY/4-PY from NMN differs and varies among species

(Garattini *et al.*, 2008; Garattini & Terao, 2011; Kitamura *et al.*, 2008; Kitamura *et al.*, 2006). Unfortunately there is no available commercial standard of 2-PY and 4-PY to confirm the identity of these metabolite peaks and due to the low concentration of these no results were obtained by LC-MS. The *mal-1* and *mal-f1* strains showed no oxidation of NMN even after extended time incubation, supporting the hypothesis that the two metabolite peaks were generated by AO mediated catalysis. In agreement with the present result Reiter *et al.* 1990 found that the xanthinuria type II patients lacking both XDH and AO were unable to oxidize NMN to its metabolites. The *in vitro* metabolism of all compounds tested as substrates for AO and XOR in all *Drosophila* strains (wild type, transgenic and mutants) is summarised in the following table 36.

Table 36: Summary of the *in vitro* oxidation of molybdoflavoenzyme substrates by *Drosophila* strains.

Substrates	<i>Drosophila melanogaster</i> strains			
	Canton-S	Transgenic (T1 and T2)	<i>ry</i> ⁵⁰⁶	Maroon-like (<i>mal-1</i> and <i>mal-f1</i>)
1-methylxanthine	++	++	-	-
3-methylxanthine	-	-	-	-
7-methylxanthine	-	-	-	-
6-mercaptopurine	-	-	-	-
Allopurinol	+++	+++	++	-
<i>All-trans</i> -retinaldehyde	++	++	++	-
Cinchonine	++	++	++	-
Methotrexate	-	-	-	-
N ¹ -methylnicotinamide	++	++	++	-
Phenanthridine	++	++	++	-
Pyrazinamide	+++	+++	++	-
Pyridoxal	+++	+++	+++	-
Vanillin	+++	+++	+++	-
Xanthine	+++	+++	-	-

(+++ = high activity ++ = intermediate activity + = low activity - = no activity).

4.2. The chemoprotective role of molybdo-flavoenzymes against xenobiotic toxicity.

At present despite MFEs being classed as phase I detoxication enzymes (Amdur *et al.*, 1991; Hodgson & Smart, 2001; Pryde *et al.*, 2010) their role in chemoprotection *in vivo* has not been proven. It is an objective of this section to measure the physiological detoxification role of AO and XDH in *Drosophila melanogaster* strains (wild type, transgenic and mutants). The potential toxicants used to evaluate this included drug such as cinchonine (anti-malaria drug), pyrazinamide (tuberculosis drug), methotrexate (anti-cancer drug), 6-mercaptopurine (anti-cancer drug) and allopurinol (gout drug). Other well known AO substrates phenanthridine (environmental pollutant) and vanillin (foods and pharmaceuticals) were also tested. Methylxanthines (caffeine, theobromine, theophylline and paraxanthine) were also used to test the detoxificative role of XDH. Finally the endogenous vitamin metabolite N¹-methylnicotinamide was also evaluated as a toxic agent to the different *Drosophila* strains.

4.2.1. *In vivo* metabolism and toxicity of methylxanthines on *D. melanogaster* strains

After 4 days of treatment with caffeine, theobromine, theophylline and paraxanthine samples of the wild type and transgenic strains did not show any known caffeine metabolites (Figures 49 and 50). Both Canton-S and transgenic flies showed a similar profile. It can be seen in figure 48 that when the strains are exposed to 20 mM of caffeine, theophylline and theobromine there was a difference in the survival times of different strains. Mutant strains (*ry*⁵⁰⁶, *mal-1* and *mal-f1*) had a higher sensitivity to the toxic methylxanthines than wild type (Canton-S) and transgenically rescued (T1 and T2) strains. Because all of the *Drosophila* strains were not affected by a dose of 20 mM paraxanthine, a higher dose of 100 mM was given to observe effects for

mutant strains (Figure 48). There was no difference between the Canton-S and the transgenic T2 and T1 strains of *Drosophila* which suggests that the transgenic *ry* strains had been rescued by the insertion of *ry* gene. This also demonstrated that it was the XDH gene that was conferring protection as mutant *ry*⁵⁰⁶ had a much reduced survival time. All toxicity results show that the mutant strains (*ry*⁵⁰⁶, *mal-1* and *mal-fl*) have a higher sensitivity to the toxic methylxanthines than wild type (Canton-S) and transgenically rescued (T1 and T2). The lifespan of mutant (*ry*⁵⁰⁶ and maroon-like) strains were reduced to 9 days when exposed to 100 mM of paraxanthine, but wild type and transgenic rescued strains were unaffected by this treatment. With regards the biochemical basis of the results obtained, caffeine and theophylline, theobromine and paraxanthine ingested are partly broken down into its metabolites by the cytochrome P4501A2 (CYP1A2), N-acetyltransferase and XDH enzymes (Rasmussen & Brosen, 1996). So, without XDH, the maroon-like and *ry*⁵⁰⁶ strains cannot fully catabolise the caffeine metabolites as the missing enzyme converts the methyl-xanthines to the more polar methyl-uric acids in the final steps of caffeine degradation. The mutant strains were affected more by the absence of the XDH, but the wild type and transgenic strains are still affected by the 20 mM caffeine and theophylline and all flies were dead after 13 days. By comparing the toxicity of methylxanthines treated with wild type and transgenic *Drosophila* strains it was found that caffeine (20 mM; Figure 59) has a greater toxicity than theophylline (20mM; Figure 59). This agrees with Zimmering *et al.* 1977 that found that caffeine is lethal to adult *D. melanogaster* (Zimmering *et al.*, 1977) and at lower concentration decreases longevity and fecundity in *Drosophila prosaltans* (Carrillo & Gibson, 2002; Itoyama *et al.*, 1998). Graf & Wurgler (1986) found that caffeine media with 4% w/v killed approximately 75% of exposed flies. Zimmering *et al.* (1977) found a high mortality

rate in the *ry*⁵⁰⁶ flies after three days exposure to 1.25-2.5 mg/ml caffeine. Zimmering *et al.*, (1977) found that the Canton-S strains mortality rate was around 40% after three days, which is higher than the results obtained from the current toxicity test (40% after 8 days). From the *ry*⁵⁰⁶ strain used in the Zimmering *et al.* (1977) study the mortality rate was almost 100% after three days, which is slightly higher than this current toxicity test found (100% dead after 6 days). This result confirms the fact that caffeine is not only mutagenic but it also causes a reduction in lifespan (Kuhlmann *et al.*, 1968). Nikitin *et al.* 2008 showed that the lifespan of Canton-S *Drosophila melanogaster* males is reduced with varying caffeine concentration (1.5-13 mM) (Nikitin *et al.*, 2008). However, theobromine (20 mM) is much less active than caffeine or theophylline (Tarka, 1982), with regard to the above results (Figure 59). Similarly paraxanthine showed a weak toxicity and wild type and transgenic strains were only affected by a higher dose of paraxanthine (100 mM; Figure 48). Czok 1974 reported the action of the theobromine in the central nervous system is weak or virtually absent (Czok, 1974), which agrees with the results obtained from the current toxicity test (20 mM theobromine had no effect in Canton-S and transgenic strains during experiment). The literature is devoid of any reference to human toxicity data on theobromine, except one general statement that “in large doses” theobromine may cause nausea and anorexia (Pharmacopeia, 1984). Comparative results for the acute toxicity of caffeine, theophylline and theobromine given orally for mouse and rat, it is of interest to note a very similar acute toxicity for caffeine and theophylline in the rat whereas theobromine was appeared to have no toxicity (Stravic, 1988; Tarka, 1982). These results agree with the results obtained from the current toxicity study. Paraxanthine, although not found in plants or foods, is a major metabolite of caffeine in humans (Aranda *et al.*, 1983). There is little information in the literature concerning

its toxicological potential, but the available information indicates that its toxicological potency in man is very low (Stravic, 1988). In spite of this, there is surprisingly little information in the literature about the toxicological potential of paraxanthine, especially in humans. A number of major reference books, including the *Merck Index* (10th Ed.) and *Martindale-The Extra Pharmacopoeia* (1982), do not list paraxanthine (Stravic, 1988). These results agree with the paraxanthine results obtained from the current toxicity study in that 100 mM paraxanthine did not have an effect on the Canton-S and transgenic strains (shown figure 59). In conclusion, it may be the specific locations of methyl groups on the purine ring are important determinants on the biological effects of these compounds. Because theobromine (3, 7-dimethylxanthine) and paraxanthine (1, 7-dimethylxanthine) were not toxic to Canton-S and transgenic strains, but caffeine (1, 3, 7 trimethyl-xanthine) and theophylline (1, 3-dimethylxanthine) were toxic, these findings may be comparable to the results of Ho *et al.* who reported that several purines such purine, adenine, 2,6 purine, xanthine and guanine have different effects on the survival rate of *Drosophila melanogaster* related to the specific location of amino and carboxyl groups, because of these positions on the purine ring were important determinants of the biological effects of these compounds (Ho *et al.*, 1984). In the study detailed in this thesis it was concluded that the methyl groups in the number 1 and 3 positions (theophylline) was more critical than the number 3 and 7 positions (theobromine), while the number 1 and 7 positions (paraxanthine) was not toxic to wild type (Canton-S) strain. The three positions of methyl groups in purine ring of caffeine (1,3,7-trimethylxanthine) was critical to survival of Canton-S strain. Overall the results of the studies here show that the loss of AO and/or XOR activity combined (*ma-l* strains) had greater effect on the ability of *Drosophila* to survive in the methylxanthine medias than the deficiency of

XOR alone (*ry*⁵⁰⁶ strain). This suggests that AO has either an involvement in the metabolism of methylxanthines or secondary protective effect.

4.2.2. *In vivo* biotransformation and toxicity of allopurinol on *D. melanogaster* strains

Canton-S and transgenic T1 and T2 generated oxipurinol (Figure 52) when treated with allopurinol. Allopurinol also caused an approximately 50% decrease in uric acid production and a significant increase in xanthine and hypoxanthine to approximately 75% and 25% respectively when compared them with untreated Canton-S. This resembles the situation in humans and rats where oxipurinol inhibits XOR which is responsible for the oxidation of hypoxanthine to xanthine and of xanthine to uric acid (Figure 52). These results are also consistent with Al Bratty *et al* who found that feeding wild type (Oregon-R) flies on allopurinol medium for 3 days, caused the level of xanthine and hypoxanthine to increased and uric acid levels dropped (Al Bratty *et al.*, 2011).

In previous studies in rats and humans, it has been suggested that both AO and XOR may oxidise allopurinol to oxipurinol based on *in vitro* data with purified enzymes and *in vivo* data with hereditary xanthinuria type I and II patients (Moriwaki *et al.*, 1993; Yamamoto *et al.*, 1991). In the present study the *ry*⁵⁰⁶ mutant strain that lack XDH activity and has AO activity, (which represents the classical xanthinuria type I due to XOR gene deficiency) and maroon-like strains (*mal-1* and *mal-fl*) due to lack of both XOR and AO activity, (which resemble classical xanthinuria type II) were treated with allopurinol and analysed by HPLC. It was found in this study that the *ry*⁵⁰⁶ strain that has AO is able to convert allopurinol to oxipurinol (see figure 52) whereas *ma-l* strains did not generate oxipurinol both *in vitro* and *in vivo* (Table 24). This

demonstrated that like mammalian AO the corresponding *Drosophila* enzyme contributes to oxipurinol oxidation.

The percentage survival in all strains for allopurinol and oxipurinol treated flies can be seen in figure 51. When exposed to 20 mM of allopurinol all the maroon-like flies were dead after 9 days. In contrast the *ry*⁵⁰⁶ strain behaved similarly to the wild type and transgenic strains when exposed to 20 mM of allopurinol in that they all survived in this concentration. Canton-S, transgenic and *ry*⁵⁰⁶ strains were treated with a higher 100 mM dosage of allopurinol. This concentration was more toxic to the *ry*⁵⁰⁶ strain indicating XDH involvement in protection against allopurinol toxicity (Figure 51). *Ma-l* strains with a combined deficiency of AO and XOR had a reduced ability to survive allopurinol toxicity indicating the additional importance of AO in protecting against this toxicant. In conclusion the results clearly show that AO and XOR protect against allopurinol toxicity in *Drosophila melanogaster*.

4.2.3. *In vivo* biotransformation and toxicity of 6-mercaptopurine on *D. melanogaster* strains

6-mercaptopurine (6-MP) is toxic to rats and mice (Higuchi *et al.*, 1978; Tapner *et al.*, 2004). Interestingly, 6-MP was not metabolised *in vitro* by Canton-S, transgenic and *ry*⁵⁰⁶ strains. On comparing the percentage survival of wild type and transgenic strains with *ry*⁵⁰⁶ and *mal-1* and *mal-fl* strains, there was no statistically significant difference between them when they were administered 20 mM 6-MP in the media (Figure 51). It can be concluded that AO and XOR in *Drosophila* do not have a role in 6-MP metabolism and thus there was no involvement in any chemoprotection against this toxic anticancer drug.

4.2.4. *In vivo* biotransformation and toxicity of cinchonine on *D. melanogaster* strains

The wild type and *ry*⁵⁰⁶ mutant of *Drosophila melanogaster* treated with cinchonine showed a metabolite peak in the chromatograms (Figure 54). In theory cinchonine may be oxidised to its metabolite 2'-cinchoninone by *Drosophila* AO as has been observed with other species such as hamster, rabbit, guinea pig and man (Al-Tayib, 2009; Beedham *et al.*, 1992; Palmer *et al.*, 1969). Because its metabolite 2'-cinchoninone is not commercially available; LC-MS was used check the identity of the cinchonine metabolite. Unfortunately due to the low level of 2'-cinchoninone in the complex biological matrix no result was obtained to confirm its identity. There was no conversion of cinchonine to the putative 2'-cinchoninone peak for maroon-like strains. The fact that there was no metabolite in *ma-l* strains suggest that the metabolite generated by the other AO-active strains is due to AO catalysis. This result also suggests that *Drosophila* AO has a greater contribution to the overall metabolic profile of cinchonine than microsomal mono-oxygenases; otherwise the metabolite peak would be found in maroon-like strains. In the present research the effect of cinchonine toxicity on wild type, transgenic and mutant strains of *Drosophila* was observed as shown in figure 53. The results indicated that the cinchonine had a high potential toxicity and caused the faster death of the maroon-like strains than wild type, *ry*⁵⁰⁶ and transgenic strains. This is presumably because the AO-deficient maroon-like strains flies are unable to metabolise the cinchonine.

4.2.5. *In vivo* biotransformation and toxicity of phenanthridine on *D. melanogaster* strains

All *Drosophila* strains were kept on media containing phenanthridine and fly extracts were analysed by HPLC. Both wild type (Canton-S) and *ry*⁵⁰⁶ strains generated the metabolite of 6(5H)-phenanthridone (Figure 54). This result is not surprising because

of the several papers reported that the phenanthridine is a good substrate and has a very high affinity towards AO (Beedham, 2001; Rashidi, 1996; Stubley & Stell, 1980). In contrast, the results obtained from this research showed that the *mal-1* and *mal-f1* strains of *Drosophila melanogaster* that are deficient in AO and XDH could not generate 6(5H)-phenanthridone. Due to the detoxification effect of AO being present Canton-S, transgenic and *ry*⁵⁰⁶ flies were less affected than maroon-like strains by phenanthridine toxicity due to the presence of AO as shown in figure 64.

4.2.6. *In vivo* biotransformation and toxicity of pyrazinamide on *D. melanogaster* strains

Pyrazinamide a drug used to treat tuberculosis is extensively metabolised *in vivo*. The main steps of pyrazinamide metabolism, which take place in mammalian liver, involves enzymatic deamidation, followed by formation of the main active metabolite, 2-pyrazinoic acid (Mehmedagic *et al.*, 2002) (Figure 61). This acid is further oxidized by the action of XOR to 5-hydroxypyrazinoic acid, the main excretory metabolite of pyrazinamide. Direct oxidation of pyrazinamide by XOR is also possible, leading to the formation of 5-hydroxypyrazinamide (Figure 61) (Kitamura *et al.*, 2006; Moriwaki *et al.*, 1997). More than 50% of pyrazinamide metabolism is mediated by XOR (Mehmedagic *et al.*, 2002). All samples of *Drosophila melanogaster* strains treated with pyrazinamide showed similar profiles that did not show any clear peaks of metabolites. Two HPLC methods (isocratic and gradient) were used in the *in vitro* study to confirm that there were no metabolites generated from *in vivo* pyrazinamide metabolism by Canton-S and *ry*⁵⁰⁶ strains (data not shown). Interestingly, although pyrazinamide is not metabolised *in vivo* by *Drosophila* it was converted to 5-hydroxypyrazinamide on *in vitro* incubations with *Drosophila* cytosol. This is may be due to the concentration dose and/or incomplete absorption in *Drosophila* or that it is

very rapidly metabolised and excreted. However, this might not be the only reason for the difference in activity of AO and XOR that was observed. The discrepancy between the *in vitro* findings compared with the *in vivo* situation in *Drosophila melanogaster* might be due to the fact that the metabolism may have undergone a different metabolic pathway, which may have given other metabolites.

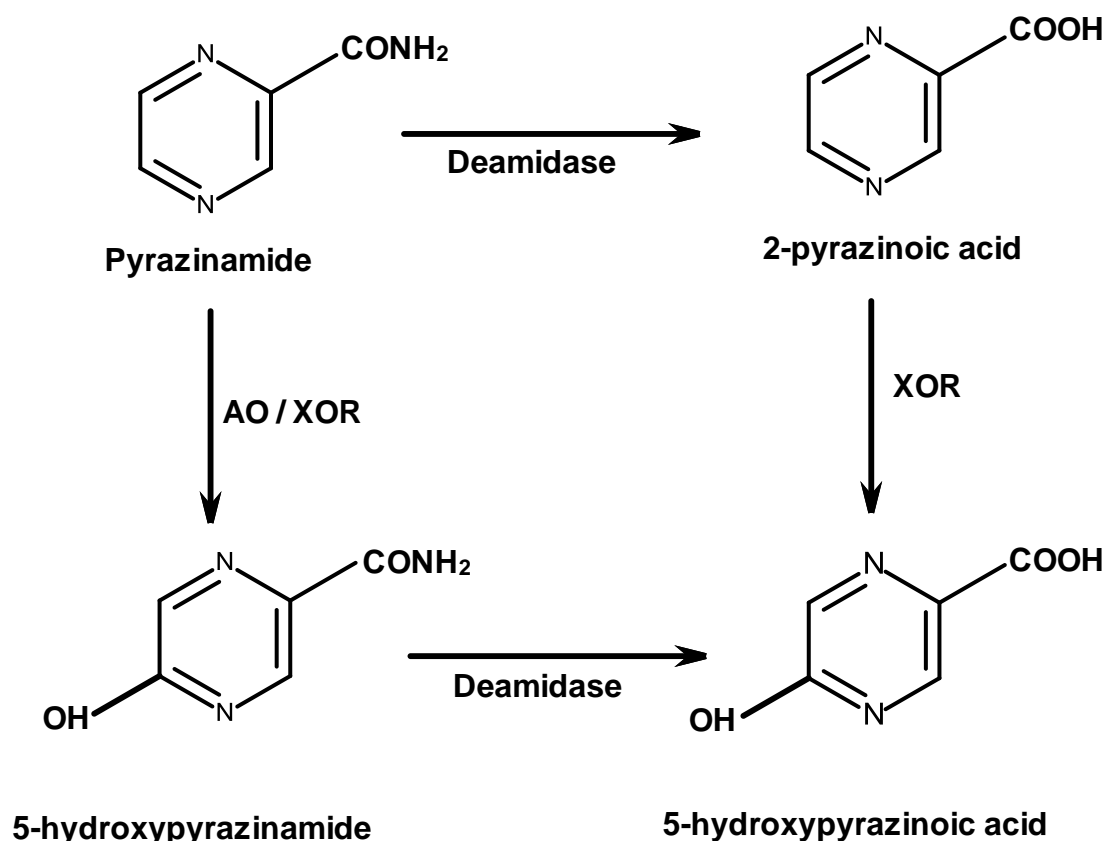


Figure 71: The principal metabolic pathways of pyrazinamide in mammals (Kitamura *et al.*, 2006; Lacroix *et al.*, 1989).

This drug was also investigated to discover any toxic effects in the different *Drosophila* strains. The results of flies fed on media containing 20 mM pyrazinamide are shown in figure 53. In terms of Canton-S and transgenic strains there was no effect by pyrazinamide, but mutant strains (ry^{506} , *mal-1* and *mal-f1*) were affected by pyrazinamide toxicity and the mortality starts from the early days of exposure,

probably due to the absence of XOR or AO/XOR enzymes and lack of any oxidation products *in vitro* studies.

4.2.7. *In vivo* biotransformation and toxicity of N¹-methylnicotinamide on *D. melanogaster* strains

In this study, N¹-methylnicotinamide (NMN) was fed to the different strains of *Drosophila melanogaster*. All samples of *Drosophila melanogaster* strains treated with NMN did not show any clear peaks of metabolites using two HPLC methods as described in materials and methods chapter. Interestingly, although NMN is not metabolised *in vivo* by *Drosophila* it was biotransformed *in vitro* by *Drosophila* (section 3.2.5). This is may be due to the dose concentration and incomplete absorption in *Drosophila* or distribution of NMN in the fly media. However, the concentration of NMN in the fly media cannot to be the only reason for the difference in activity of AO that was observed. Alternatively, the metabolism may have undergone a different metabolic pathway. The results observed from the NMN toxicity tests as seen in figure 55 shows that the maroon-like strains of *Drosophila* lacking AO is less able to survive in media containing NMN. This is presumably because the flies are unable to metabolise NMN as they lack AO. Although the toxicity of N¹-methylnicotinamide has not been previously reported in any organism it has been reported that nicotinamide does have a very low acute toxicity to mammals (Florin *et al.*, 1980).

4.2.8. *In vivo* biotransformation and toxicity of vanillin on *D. melanogaster* strains

Canton-S, transgenic and *ry*⁵⁰⁶ strains treated with vanillin generated its metabolite vanillic acid (Figure 59). These results proved that vanillin is converted into vanillic acid by AO because the *ry*⁵⁰⁶ strains only have AO and not XDH. The present study

therefore confirmed that vanillin is a good substrate of *Drosophila* AO as it is in mammals (Panoutsopoulos *et al.*, 2005). HPLC analysis of maroon-like strains revealed a lack of any detectable vanillic acid peak, with *ma-l* strains (Figure 59). This further confirmed AO is the major route of vanillin oxidation *in vivo* in *Drosophila*. For toxicity tests *Drosophila* from each strain were exposed to vanillin in low and high concentration (20 mM and 100 mM). The results showed no toxicity with all strains of *Drosophila* when exposed to the 20 mM of vanillin (Figures 56). However, the effects of vanillin observed at a five times higher concentration (100 mM) in strains that harbouring mutations in MCS genes (*mal-1* and *mal-fl*) had an increased sensitivity when treated with the high concentration of vanillin (shown figure 58). This may be because the vanillin dosage was high enough to compromise this strains ability to detoxify this chemical. This indicated that AO in *Drosophila* serves to protect the organism from the toxic effects of high vanillin concentration.

4.2.9. Summary of enzyme assays, *in vivo* metabolism and toxicity of compounds in *Drosophila* strains.

A summary of the toxicity, *in vivo* and *in vitro* metabolism of all compounds tested in wild type, transgenic and mutant of *Drosophila melanogaster* treated with compounds is shown in table 37.

Table 37: Summary of the toxicity, *in vivo* and *in vitro* oxidation of purines, non purines, N-heterocyclic and aldehyde substrates by *Drosophila* strains used in this study.

AO/ XDH substrates		<i>Drosophila melanogaster</i> strains			
		Canton-S	Transgenic (T1 and T2)	<i>ry</i> ⁵⁰⁶	Maroon-like
Allopurinol (AO and XDH)	<i>In vivo</i> metabolite	+++	+++	++	-
	<i>In vitro</i> metabolite	+++	+++	++	-
	Toxicity	-	-	-	+++++
Caffeine (XDH)	<i>In vivo</i> metabolite	-	-	-	-
	Toxicity	+++	+++	+++++	+++++
Cinchonine (AO)	<i>In vivo</i> metabolite	++	++	++	-
	<i>In vitro</i> metabolite	++	++	++	-
	Toxicity	+++	+++	+++	+++++
N¹-methylnicotinamide (AO)	<i>In vivo</i> metabolite	-	-	-	-
	<i>In vitro</i> metabolite	++	++	++	-
	Toxicity	-	-	-	+++++
6-mercaptopurine (AO and XDH)	<i>In vivo</i> metabolite	-	-	-	-
	<i>In vitro</i> metabolite	-	-	-	-
	Toxicity	+++	+++	+++	+++++
Paraxanthine (XDH)	<i>In vivo</i> metabolite	-	-	-	-
	Toxicity	+	+	+++++	+++++
Phenanthridine (AO)	<i>In vivo</i> metabolite	++	++	++	-
	<i>In vitro</i> metabolite	++	++	++	-
	Toxicity	+	+	+	+++++
Pyrazinamide (AO and XDH)	<i>In vivo</i> metabolite	-	-	-	-
	<i>In vitro</i> metabolite	+++	+++	++	-
	Toxicity	+	+	+	+++++
Theobromine (XDH)	<i>In vivo</i> metabolite	-	-	-	-
	Toxicity	++	++	+++++	+++++
Theophylline (XDH)	<i>In vivo</i> metabolite	-	-	-	-
	Toxicity	+++	+++	+++++	+++++
Vanillin (AO)	<i>In vivo</i> metabolite	+++	+++	+++	-
	<i>In vitro</i> metabolite	++	++	+++	-
	Toxicity	-	-	-	++

(+++++ = extremely high activity or toxicity, +++ = high activity or toxicity, ++ = intermediate activity or toxicity, + = low activity or toxicity, - = no activity or no toxicity).

The difference observed between the mutant strains, transgenic and wild type strains supports the conclusion that lack of MFEs is responsible for the strains reduced survival in the presence of toxin. The caffeine, theobromine, theophylline and 6-mercaptopurine had similar effects as these compounds were not biotransformed by *Drosophila* molybdo-flavoenzymes. In contrast, the allopurinol, vanillin, N¹-methylnicotinamide, phenanthridine and pyrazinamide which are substrates for AO and/or XOR had no or less toxic effect on the wild type and transgenic strains due to these substrates being *in vitro* and *in vivo* biotransformed by AO and/or XOR in

Drosophila (Table 37). A summary of the relative toxicity of each compound is shown in table 38.

Table 38: Summary of the toxicity of purines based, purines analog, N-heterocycles and aldehyde compounds to *Drosophila melanogaster* strains.

Compound	50% mortality (days)				100% mortality (days)			
	Canton-S	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-f1</i>	Canton-S	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-f1</i>
Allopurinol (20mM)	None	None	4	4	None	None	9	9
Allopurinol (100mM)	None	10	----	----	None	15	----	----
Caffeine (20mM)	9	3	2	2	13	8	6	6
Cinchonine (20mM)	4	3	2	2	8	8	5	5
6-mercaptopurine (20mM)	7	6	4	4	15	13	9	7
N ¹ -Methylnicotinamide (20mM)	None	None	4	4	None	None	9	9
Paraxanthine (100mM)	None	4	4	4	None	9	9	9
Phenanthridine (20m)	None	None	6	6	None	None	12	13
Pyrazinamide (20 mM)	None	5	5	5	None	12	8	8
Theobromine (20mM)	None	3	3	3	None	8	8	8
Theophylline (20mM)	8	4	4	4	13	10	7	8
Vanillin (100mM)	None	None	8	8	None	None	19	18

None: not affected

----: not tested

Yellow: 2-7days, blue: 8-19 days and green: None.

As seen in table 38 it was observed that of the compounds tested the anti-malarial alkaloid cinchonine was the most toxic of the compounds tested, killing wild type and *ry*⁵⁰⁶ flies within 7 days and maroon-like strains within 5 days of administration of a 20 mM concentration in media. This may be due to the fact that it has more efficient properties in increasing the intracellular cytotoxicity (Genne *et al.*, 1994). The second most potent class were alkylated purine drugs, caffeine and theophylline with wild type strain dying within 13 days of administration. It was determined that the maximum life of mutant (*ry*⁵⁰⁶ and *ma-l*) flies was 8 days of administration of a 20 mM concentration of caffeine and theophylline in media (Table 38). Theobromine was

more toxic with ry^{506} and maroon-like strains dying within 8 days, but it had no lethality at 20 mM concentration with wild type strain. The third-most toxic compound tested was 6-mercaptopurine (anti-cancer drug), which reduces lifespan in wild type, ry^{506} and maroon-like strains to 16 days, 13 days and 7 days respectively. Allopurinol and paraxanthine had no lethality at low and higher doses of 20 and 100 mM respectively (allopurinol and paraxanthine) with wild type strain. The food constituent, vanillin and the urban air pollutant phenanthridine had no toxicity at any dose tested for wild type and ry^{506} strains. The anti-tuberculosis drug, pyrazinamide had no toxicity at 20 mM tested for wild type strain. In comparison, a great toxicity effect was observed on the lifespan of the maroon-like strains (*mal-1* and *mal-f1*) when exposed to the toxicity of purines, purines analogs, N-heterocycles and aldehyde compounds (Table 38), that are AO substrates indicating its essential role in chemoprotection *in vivo*.

4.3. The effect of molybdo-flavoenzyme deficiencies on lifespan of *Drosophila melanogaster*.

D. melanogaster is a powerful model to study lifespan and aging (Lim *et al.*, 2006; Shaw *et al.*, 2008) and its lifespan is approximately 60 days (Shaw *et al.*, 2000). Antioxidants that help reduce free radical damage have been shown to have an effect on the longevity of *Drosophila* (Le Bourg, 2001). Uric acid which has antioxidant characteristics has been observed to increase the longevity of flies (Le Bourg, 2001). As XOR plays an important role in the generation of uric acid, a known antioxidant, it has been hypothesised that it may play an important role against ageing and therefore increases the lifespan of individuals (Ames *et al.*, 1981; Cutler, 1984; Hediger, 2002). XOR-deficient ry^{506} flies are a good model for testing this hypothesis. It was also decided to investigate the effects mutations in the MCS gene have on lifespan that

have a dual deficiency of AO and XOR. In the study presented in this thesis, to confirm the level of uric acid in all *Drosophila* strains, the extracts of all strains were analysed by HPLC. HPLC profiles for all *Drosophila* strains are shown in figure 58. Comparison of the endogenous purine levels of mutant *Drosophila* strains (ry^{506} and maroon-like) (Table 27) with the normal *Drosophila* strain (Canton-S) indicated that the xanthine to hypoxanthine ratio was approximately 4 times the level of normal fly which is similar to the situation in human patients with severe hereditary xanthinuria (Simmonds, 2003; Simmonds *et al.*, 1995) (Table 27). The xanthine level of this mutant was 7 fold higher than in the normal strain, but no difference in the hypoxanthine level was seen. Mutant strains (ry^{506} and maroon-like) had no detectable uric acid demonstrating that these strains mimic human hereditary xanthinuria (Table 27). The total absence of uric acid in the three mutant strains also demonstrated that the XOR enzyme was totally inactive. These results were consistent with Hilliker *et al.* and Kamleh *et al.* who found that ry^{506} mutants are unable to make uric acid (Hilliker *et al.*, 1992; Kamleh *et al.*, 2008). The effect of the lack of XOR and AO has on the longevity of *Drosophila* wild type, transgenic, ry^{506} and *ma-l* strains are shown in figure 60. The ry^{506} strain had a mean lifespan that was approximately 60% that of the Canton-S strain. Maroon-like flies (*mal-l* and *mal-fl*) had a considerably decreased lifespan compared with Canton-S and ry^{506} strains respectively, with their lifespan being only approximately 30% and 60% of these strains respectively. The ry^{506} strains that have had the *ry* gene re-inserted by transgenesis (T1) had a lifespan which is similar to that of the Canton-S strain lifespan. The difference observed between the wild type and mutant strains (Table 28) confirms the observation of Shepherd *et al.* (1989) who found that a mutant ry^{506} strain lacking XOR activity had a reduced lifespan compared to the wild type strain (Shepherd *et al.*, 1989). As seen in the table

32, it was observed that the maximum lifespan of the wild type strain was 65 days, this is consistent with Ayar *et al.*, (2009) who found that the maximum lifespan of wild type strain was 70 days (Ayar *et al.*, 2009). The results observed during longevity studies carried out under normal conditions reflect the observation that uric acid has antioxidant properties that protect against free radicals and therefore extend an organism's longevity. This finding also agrees with studies, which found that the urate-null strains of *Drosophila* are sensitive to oxidative stress (Hamatake *et al.*, 2009; Hilliker *et al.*, 1992). The results in this thesis agree with the study of Kim *et al.* (2001) who found the ry^{506} mutants had a reduced lifespan that was 40% of that found in the wild type strain. Along with a reduction in lifespan the ry^{506} flies also had a reduced immune response to bacterial infection when compared to a wild type strain (Kim *et al.*, 2001).

The results obtained demonstrated that the lack of AO activity along with XOR activity has greater effect on the ability to survive than the loss of only XOR activity. This was surprising, because AO does not generate or remove any known endogenous chemicals that are at present known to affect lifespan. From the *in vitro* experiments detailed in section 3.2 the AO-deficient maroon-like strains could not metabolise several endogenous vitamins, namely vitamin A, B3 and B6 so perhaps this inability may affect lifespan due to accumulation of precursors or lack of generation of the metabolites. Obviously further experiments would be required to prove this hypothesis (see toxicity section 2.4).

4.4. The effect of heat and cold shock on wild type and MFE-deficient *Drosophila* strains.

To determine whether or not the lack of these enzymes have any effect on the flies defences against extreme temperatures flies were exposed to cold and heat shock treatments.

4.4.1. Effect of 25, 37 and 39 °C heat shock on *Drosophila melanogaster* strains.

The relationship between longevity and ageing of insects and stress factors have been known for some time (Vermeulen & Loeschke, 2007). An insect's survival depends on its ability to survive different types of environmental stress which include exposure to high and low temperatures. Cells continuously exposed to either exogenous or endogenous stress such as heat or radiation have developed variety protective mechanisms such as the synthesis of stress proteins, antioxidant defence or DNA repair enzymes (Bochner *et al.*, 1984; Niedzwiecki *et al.*, 1992). The similarity of response to elevated temperatures and various oxidative stresses in bacteria (Christman *et al.*, 1985), plants (Czarnecka *et al.*, 1984) and *Drosophila* (Ashburner & Bonner, 1979) suggest the possibility that increased temperature and oxidative stress may be related. Exposure to heat is accompanied by an elevation in oxygen consumption. Thus, exposure to high temperature most likely results in an increased production of free radicals, which generate the need for an elevated level of scavenging antioxidant enzymes to remove these toxic species (Niedzwiecki *et al.*, 1992). Though an effective antioxidant-scavenging system is necessary during normal metabolic conditions to protect cellular components against oxidative damage, the need for this protection increases during periods of stress, such as exposure to heat (Niedzwiecki *et al.*, 1992; Yano *et al.*, 1987). In this study, the effects of heat shock on longevity were studied in wild type *Drosophila* and mutants lacking the antioxidant uric acid.

Figure 61 shows the mean percentage survival for all strains at 37°C for various durations (1, 2, 4 and 6 hours). All strains had no effect when exposed to heat shock at 37°C for 2 hours. All the Canton-S flies survived at 4 hours but had a relatively high mortality rate at 6 hours (approximately 50%) at 37°C, whereas the rosy strain had a significantly higher mortality rate after four hours (approximately 30%) and after six hours was approximately 60%. The heat shock at 37°C also had a significant effect on the mortality of maroon-like strains; the survival rate of *mal-1* and *mal-fl* strains was approximately 40% at 4 hours and all the flies were dead at six hours. The highest temperature of 39°C at four and six hours found none of maroon-like strains survived. The heat shock at 39°C results show that the *ry*⁵⁰⁶ strain had a significantly reduced percentage survival after four and six hours compared to the wild type Canton-S strain (shown figure 61). At 39°C the rosy mutant strain at two hours appears to have a low percentage survival (approximately 60%) and *mal-1* and *mal-fl* strains at the same duration appear to have a highly significant death levels with mortality being approximately 90% when, compared with Canton-S strain (approximately 20%). The heat shock results show that the rosy and maroon-like strains have a significantly reduced percentage survival after four and six hours when compared to the wild type Canton-S. With the maroon-like strains, all flies were dead after 6 hrs when exposed to heat shock both at 37°C and after 4 and 6 hrs at 39°C. This leads to the conclusion that the lack of AO activity along with XOR activity has greater effect on the ability to survive during extreme environmental changes than the lack of only XOR activity. The heat shock itself would cause environmental stress, raising the amount of free radicals, thus without XDH no uric acid would be protecting from these free radicals as antioxidant. The molecular basis of additive effect on mortality due to the lack of AO is unknown.

4.4.2. Effect of 0 and -3 °C cold shock on *Drosophila melanogaster* strains.

Insects living along altitudinal and latitudinal gradients survive better in lower than in higher temperatures (Anderson *et al.*, 2005). Subzero temperatures can be deadly for various insect species including *D. melanogaster*. *D. melanogaster* populations from cold climates are more cold tolerant (Guerra *et al.*, 2000). In many studies, some stress factors (i.e.: heat or cold shock) when applied at a low rate and in a short time show an hormetic effect in *Drosophila*, but when intensity and time of application were increased, it was observed that there were harmful effects (Ayar *et al.*, 2009; Le Bourg, 2007). In earlier studies, direct application of cold shock brings about sudden structural changes on protein and cell membranes in *Drosophila* (Kelty & Lee, 1999). Induction of stress protein cryoprotectants may also play a role in the cold shock response in some insects, while the importance of these factors is less clear in the case of *D. melanogaster* (Chen & Denlinger, 1992; Kelty & Lee, 2001; Nielsen *et al.*, 2005; Qin *et al.*, 2005). Death after a cold shock is not due to the cold shock, but is linked to the loss of membrane function due to depolarization of the cell membrane, since the ultimate freezing point of *D. melanogaster* is -20°C (Overgaard *et al.*, 2005). Cold shock and freezing also induce apoptosis (programmed cell death) (Yi *et al.*, 2007). In addition cold shock causes insects to lose motor activity in a reversible state known as chill coma. Although the mechanisms of the chill coma onset are poorly understood, Goller and Esch (1990) suggest that it results from a loss of function of the ion channels necessary for maintaining the membrane potential, leading to voltage equilibration and a loss of muscle cell excitability (Goller & Esch, 1990). *D. melanogaster* exposed to cold shock express Hsp70, not from the cold shock, but as a result of heating up during recovery from the effects of the cold shock (Overgaard *et al.*, 2005; Sejerkilde *et al.*, 2003).

The results obtained when wild type and mutant strains were exposed to cold shock temperatures (0 and -3°C) for different periods (1, 2, 4, and 6 hrs) are summarised in figure 62. There was no significant effect between strains when were exposed to cold shock at 0°C for 1 hr. However, between 2 and 6 hrs at 0°C cold shock there was steady decrease in percentage survival to approximately 60% for Canton-S strain, while for the period between 1 and 6 hrs the *ry*⁵⁰⁶, *mal-1* and *mal-f1* strains had a significantly high mortality rate with approximately 85% of rosy and 100% of maroon-like strains dying (Figure 62). The results of -3°C cold shock temperature experiment are presented in figure 66. No significant differences were observed between CS and rosy strains at -3°C degree for 1 hr. The survival percentage of *ry*⁵⁰⁶ and maroon-like strains showed a highly significantly mortality rate for these strains from 1 to 4 hrs periods, while all flies are dead after 4hrs. -3°C cold shock temperature had a significant and large effect on the mean percentage survival of Canton-S strain at 4 hrs and all flies were dead after 6 hrs (Figure 62). These findings are consistent with Duncker *et al.* 1995 results. Duncker serendipitously demonstrated the protection role of XDH in cold stress. Duncker *et al.* (1995) incubated non-transgenic adult *D. melanogaster* P[ry,YP 1, 2: wAFP²; YP 1-3]3 without antifreeze proteins gene (AFPs) gene as control, transgenic flies (*Ifm(3)3 ry*⁺) with AFPs gene and mutant flies (*Ifm(3)3 ry*⁸) (uric-null or non-Xdh gene) with AFPs gene as a marker gene to identify it is important for the improved cold tolerance in ice (0°C) and low temperature water bath (-7°C) for long period (24-34 hours) (Duncker *et al.*, 1995). They found that all transgenic strains demonstrated higher survival rates than (*Ifm(3)3 ry*⁸) (uric-null) mutant. It was hypothesised that the cold shock protection might be due to the Xdh genes role. This would seem to support the idea that the mutant strains

which do not contain the XDH uric acid producing enzyme have a reduced antioxidant capability that might protect them against cold stress.

4.5. Identification of mutations in the MCS Gene in *mal-1* mutants.

The cloning and sequencing of the entire MCS gene of *Drosophila* strains identified several nucleotide differences between the databank sequences and the strains studied. This section aims to discuss the consequence of these nucleotide variations. Nucleotide sequence variations between Canton-S (wild type) DNA and database AC01178 and AF162681 were found. Several silent variations were found in intron 1 (IVSI-93C>T, IVSI-135A>T and IVSI-151A>G), exon 3 (c.260T>A, c.808C>A and c.1196G>A) and exon 4 (c.1884A>C, c.2483A>C, c.2143C>A, c.2289G>T, c.2567C>T and c.2890C>T) (see figure 65).

4.5.1. Cloning and sequencing of the *mal-1* mutant strains.

The MCS gene was also sequenced in the maroon-like strains. When the sequence results from these mutants were aligned to the wild type strain (Canton-S) the following differences were found in PCR product DMCS-8. Alignment of the 3463 bp contig from the *mal-1* mutants with the wild type gene are shown in figures 66 and 67; this revealed mutations in exon 4 of MSC gene. In order to determine the effect of these mutations on the deduced amino acid sequence, cDNA sequences were derived from the mutant gene sequences by computerised removal of intron sequence from the mutant strains. This indicated that the mutations would result in a 6 bp insertion at positions c.1569_1573ins from the ATG codon in the predicted mRNA transcripts from the *mal-1* mutants. Translation of the predicted mRNA from the *mal-1* mutant indicated the 6 bp insertion was in-frame and resulted in 2 amino acids (p.Ile577_Tyr578insLeuPro) being inserted into the predicted protein (Figure 67). In order to determine whether the mutation affects vital domains in the MCS protein the evolutionary conservation of MCS protein was determined. This was done by deducing the protein sequence from the *mal-1* mutant strain of *Drosophila* and

comparing it to the sulphurase sequence of kingdoms of eukaryotic and metazoan, for example taxa (plant and green algae, fungi and bacteria) and taxon or phylum (sponges, protostomes, deuterostomes and chordates) obtained from the NCBI database. The mutations identified in the *mal-1* strains were then superimposed on the wild type (Canton-S) sequence to show if the mutations are found in a conserved region (Figure 72). Figure 72 identifies that the insertion of the amino acids leucine (L) and proline (P) disrupts between the conserved amino acid sequence (Y/F), P, (V/I) and (KSC). The insertion of two amino acids might affect the folding of the protein which may mean that vital active sites are no longer accessible. This along with the disruption of a region of amino acids conserved almost completely throughout evolution leads to the conclusion that the 6bp insertion observed in the *mal-1* mutant strain is responsible for the phenotype observed. This insertion in *mal-1* was found in MOSC N-terminal beta barrel domain whose exact mechanism function in MCS gene function is still unclear (Bittner *et al.*, 2001; Ping-Min *et al.*, 2009).

<i>ma-1</i> strain	QMAILPYPVKSCAA
Canton-S strain	QMAI YPVKSCAA
<i>Anopheles gambiae</i>	QLCL YPVKSCGP
<i>Bombus terrestris</i>	QLYI YPIKSCGA
<i>Camponotus floridanus</i>	ALFI YPIKSCGA
<i>Bombyx mori</i>	EICI FPIKSCGA
<i>Tribolium castaneum</i>	QIIL YPIKSCGG
<i>Acyrtosiphon pisum</i>	RLFV FPIKSCGA
Human	NLYL YPIKSCAA
Dog	NLYL YPIKSCAA
<i>Monodelphis domestica</i>	NIYI YPIKSCAA
Bovine	NLFL YPIKSCAA
<i>gallus</i>	NIYL YPIKSCSA
<i>Meleagris</i>	NIYL YPIKSCSA
<i>Danio rerio</i>	NLFI FPVKSCAS
<i>Oreochromis</i>	NIYI YPIKSCGA
<i>Medicago truncatula</i>	NLYL YPIKSCAA
<i>Oryctolagus</i>	NIYL YPIKSCAA
<i>Ornithorhynchus</i>	NIYI YPIKSCAA
<i>Taeniopygia gallopavo</i>	KIYL YPIKSCSA
<i>Amphimedon queenslandica</i>	RILL YPVKSCAG
<i>Sporisorium reilianum</i>	NITV YPIKSCAG
<i>Arabidopsis</i>	SITV YPIKSCAG
<i>Trichophyton</i>	KLCI YPIKSCGA
<i>Brachypodium</i>	SIII YPVKSCQG
<i>Glycine</i>	QMAI YPVKSCAA
<i>Oryza sativa</i>	SIII YPVKSCQG
<i>Ostreococcus tau</i>	ELVT YPVKSCAG
<i>A.nidulans</i>	SLSV YPIKSCGA
<i>Batrachochytrium</i>	AIHV YPIKSCGA

Figure 72: Alignment of MCS protein sequences from species throughout evolution.

The deduced amino acid sequence of MCS gene product was aligned with its homologs using CLUSTAL X programme (Larkin *et al.*, 2007). Amino acids shaded in grey indicate similarity. Yellow = regions conserved throughout all species. *Arabidopsis thaliana* (plant) (AY034895), human (BAA91354), cattle (*Bos taurus*, BAA98133), Canton-S (*D. melanogaster*), *ma-1* (*D. melanogaster*), *hxB* of *A. nidulans*, (AAF22564), Japanese rice (*Oryza sativa japonica*) (plant) (Q655R6), *Bombyx mori*- (insecta) (InQ8IU29) (domestic silkworm), *Gallus gallus* (chicken) (XP_419048), *Oreochromis niloticus* (Nile tilapia) (XP_003441281), *Tribolium castaneum* (beetle) (XP_967646), *Camponotus floridanus* (insecta) (EFN72786), *Danio rerio* (zebrafish) (XP_701766), *Canis lupus familiaris* (dog) (XP_547604 gene), *Amphimedon queenslandica* (sponge) (XP_003388765), *Sporisorium reilianum* SRZ2 (plant) (CBQ73967), *Batrachochytrium dendrobatidis* JAM81 (fungi) (EGF79866gene), *Glycine* (plant) (AW318329), *Meleagris gallopavo* (turkey) (XP_003204917), *Ostreococcus tauri* (green alga) (XP_003083410), *Anopheles gambiae* (insecta) (XP_310528), *Brachypodium distachyon* (plant) (XP_003563394), *Bombusterrestris* (insecta) (XP_003397325), *Monodelphis domestica* (vertebrata) (XP_001367755), *Trichophyton* CBS 118892 (XP_003232784), *Ornithorhynchus anatinus* (platypus) (XP_001506730), *Medicago truncatula* (plant) (AES73058) and *Acyrtosiphon pisum* (insecta) (NP_001191876). Amino acids highlighted in red text are those inserted in *ma-1*-strain.

4.5.2. Cloning and sequencing of the *mal-f1* mutant strains.

The same procedure above was also carried out for the maroon-like mutant *mal-f1*. Alignment of the 3463 bp contigs from the *mal-f1* mutant with the wild type gene shown in figure 68, revealed a mutation in exon 4. Comparison of the sequence data obtained from the *mal-f1* and wild type strains identified a 23 bp deletion at nucleotide positions c.2219_2220delCGTGGACATGAATGGCATGGCGT (Figure 68).

Translation of the coding region of the *mal-f1* strain resulted in a frame shift and the addition of a premature stop codon at codon position 562, 23 bp downstream of the deletion (Figure 69). In contrast to the wild type strain that has 782 amino acids the truncated protein formed in the *mal-f1* strain is ~28% shorter than wild type protein, from which are removed 221 of the 782 amino acids residues . The mutation also introduces ETLHGVVPN at the C-terminus (Figures 70 and 73). The premature stop codon introduced by the *mal-f1* mutation leads to the removal of a number of amino acid residues that are highly conserved throughout evolution, and the loss the C-terminus of the protein (Figure 73) that contains a domain for the conversion of bound desulpho-molybdenum cofactor into sulphurated molybdenum cofactor, which is required to activate XDH and AO enzymes (Bittner *et al.*, 2001; Schwarz *et al.*, 2009; Wollers *et al.*, 2008). So it is evident that this truncated protein has lost the MOSC N-terminal beta barrel and a C-terminal domain in MCS protein which may act as a scaffold protein prior to the activation of XDH and AO (Wollers *et al.*, 2008). This leads to the conclusion that the 23bp deletion observed in the *mal-f1* strain is responsible for the phenotype observed (Figure 73). Interestingly the MCS gene mutation in tomato *flacca* occurs at the C-terminus of the MoCo sulfuryase protein (Sagi *et al.*, 2002) that disrupted MoCo sulfuryase function and results in the lack of

AO and XDH activities and Aba content in plant tissues and leading to a wilted phenotype (*flacca*) (Seo *et al.*, 2004).

Figure 73: Amino acid sequence alignment of deduced *ma-l* MCS proteins and homologs in other species.

The deduced amino acid sequence of MCS gene product was aligned with its homologs using CLUSTAL X programme (Larkin *et al.*, 2007). Dotted lines indicate gaps that were introduced to maximize the alignment. Numbers on the left indicate the position of the first residue in each string. The putative PLP binding site domain is framed by red box and the conserved lysine residue in the PLP domain is indicated in red text. The conserved cysteine residue is indicated with a red asterisk. The MOSC N-terminal beta barrel domain is framed by black box. The carboxy-terminus indicated by blue box; 2Fe-2S, iron-sulfur binding region (2Fe/2S) is framed by green box. The carboxy-terminus indicated by blue box. Red text indicates amino acid sequence following deletion in *D. melanogaster mal-f1* strain. Amino acids shaded in grey indicate similarity and amino acids highlighted in red are those inserted in *D. melanogaster mal-1* strain. Yellow = regions conserved through out all species. Green = regions conserved between four and five of the species. Blue = regions conserved between three of species. Pink = regions conserved between two of species. GeneBank accession numbers are in parentheses, Ar = *Arabidopsis thaliana* (*Aba3*, AY034895), tomato (*FLACCA*) (AY074788), human (BAA91354), cattle (*MCSU* of *Bos taurus*, BAA98133), Canton-S, MCS (*D. melanogaster*), *mal-1* MCS (*D. melanogaster*), *mal-f1* MCS (*D. melanogaster*) and *Aspergillus* (*hxB* of *A. nidulans*, AAF22564).

Ar. (1) -----MEAFLEKFGDYYPGDPGPKNQEIRDTEFKRDKG-VYLDHAGSTLYSELMEYIFKDFTSNVFGNPHSQSDISSA--SDLTADARHQVLEYFNASPEDYSCFLTSGATAALKLVGETFPWTQDSN-----FLVTMENHNSVLGIRE

Tomato (1) -----MNIESEKEQFLKEFGYYGYANSKPNIDEIRATEFKRND--TYLDHAGATLYSESQMEVFKDINSTLYGNPHSQSTCSLAEDIVGKARQOVLSEFNASPREYSCIFTSGATAALKLVGETFPWSSNSS-----FMYSMENHNSVLGIRE

Human (1) MAGAAAESGRELWTFAGSRDPSAPRLAYGYGPGSLRELRARESRLAGT--VYLDHAGATLFSQSQLESFSTSDIMENTYGNPHSQNISSKLTHDTVEQVRYRLAHFHTTAEDYTVIFTAGSTAALKLVAAEAPFWVSGPPESSGSRFCYLTDSHTSVVGMRN

Cattle (1) -----MQSRQ-----RALLP-----RSPGT--VYLDHAGTTLFPQSQTFSMKDMENYVGNPHSQNISSKLTHDTVEQVRYRLAHFHTTAEDYTVIFTAGSTAALKLVAAEAPFWVSGPPESSGSRFCYLTDSHTSVVGMRN

Canton-S (1) -----MTSYRREFFS-----ASEQSQIDAESFRLASKNSTYLDHAGTTLTAAEQLOQNVICNPH-----TCRLTGDFVDQVRFKILEFFNTTAEDYHVIFATANATAALSLVAENFDGSSGSE-----FHFCQENHNSVLGMR

mal-f1 (1) -----MTSYRREFFS-----ASEQSQIDAESFRLASKNSTYLDHAGTTLTAAEQLOQNVICNPH-----TCRLTGDFVDQVRFKILEFFNTTAEDYHVIFATANATAALSLVAENFDGSSGSE-----FHFCQENHNSVLGMR

mal-1 (1) -----MTSYRREFFS-----ASEQSQIDAESFRLASKNSTYLDHAGTTLTAAEQLOQNVICNPH-----TCRLTGDFVDQVRFKILEFFNTTAEDYHVIFATANATAALSLVAENFDGSSGSE-----FHFCQENHNSVLGMR

A.nidulans (1) -----MNLKGTAAAYCSGYSSEDDVIREREPYPLKDT--TYLDHAGTTLTANSLHSGFGRDLTGNLYGNPHSMSASSQLSAQRAGRYSLRALRFFNADPDEFDLVFANATAGIKLVADALQNSPQGF-----WYGYVDAHTSLVGVR

Ar. (162) YALAQGASACAVDIEEAAQPGQLTNSGPSIKVKHRAVQMRNTSKLQKESRGNAYNLFAFPSECNFSGLRFNLDLVKLMKENTETVLQ---GFPFSKSK-----RWMVLIDAAGKCATLPPDLS---EYPADFVLSFYKLFYPTGLGALLVRNDAKLL

Tomato (167) YALSKGAAFAVDIEDTH--VGESESPQSNLKLTHQHIQRNNEGVLKGMGTNTYNLFAFPSECNFSGRKFDPNLIKKEGSEKILE---SQYRSRG-----CWLVLIDAAGKCATNPPNLS---MFTADFVLSFYKLFYPTGLGALLVRKDAKLL

Human (179) VTMAINVISIPVRPEDLW-----SAEERASAS-NPDCQL-PLHFCYPAQSNFSGVRYPLSWIEEVKS-GRIRP-----VETPGK-----WFLVLDAASVYSTPLDLS---AHQADFVLSFYKLFYPTGLGALLVHRAAPLL

Cattle (146) ITAAMNVSSIPVRPEDMW-----SAERQDAAAAGDPAPQP-PLHFCYPAQSNFSGVRYPLSWIEEVKS-GRIRP-----AARPGK-----WFLVLDAAAFVGTSPDLS---VHQADFVLSFYKLFYPTGLGALLVNNRLAALL

Canton-S (150) RVRENGIYML-RENEIS-----GGKHKANGKVHVESGKTGNSLLTFSACNFGSGYKIPLEVIEQIQIDGLAKPGKELWSLGEKKKNMHNDDYICLDAASFVATSPDLS---KYRDPYVCLSFYKLFYPTGLGALLVSRRAEVEF

mal-f1 (150) RVRENGIYML-RENEIS-----GGKHKANGKVHVESGKTGNSLLTFSACNFGSGYKIPLEVIEQIQIDGLAKPGKELWSLGEKKKNMHNDDYICLDAASFVATSPDLS---KYRDPYVCLSFYKLFYPTGLGALLVSRRAEVEF

mal-1 (150) RVRENGIYML-RENEIS-----GGKHKANGKVHVESGKTGNSLLTFSACNFGSGYKIPLEVIEQIQIDGLAKPGKELWSLGEKKKNMHNDDYICLDAASFVATSPDLS---KYRDPYVCLSFYKLFYPTGLGALLVSRRAEVEF

A.nidulans (159) LAKMGSRCFVNEDVDWSW-----ISGLSRRRESLGLFAYPAQSNMNGRRVPMRWCEQIRAKENADN-----MITYLDAASFVATSPDLSKIAAAPPFTVLVLSFYKLFYPTGLGALLVRKSSGDF

Ar. (288) KKT-YFSGGTVAASIADIDFVKRRE--RVEEFFEDGSASFSLAAIRHFKLKSLLP-----SAWMHTTS-SIVVKKLKALRHNGAAVCVLYGSENLELSHKS-GPTVTFNLKRPDGSWFGYLEVEKLASLSEIQLRITGCFNPGACAKYLEL

Tomato (307) KKT-YFSGGTVAASIADIDFVKRRE--GVEEFFEDGTSIFSLTAIQHGFKINMLT-----SSIFRHTTSIAYVRNKLALKHENGFEVCTLYG-----LLSEM-GPTVTFNMRKPDGTWYGYREVEKLATLAIQIRITGCFNPGACAKYLEL

Human (290) RKT-YFGGTAASAYLAGEDFYIPQ--SVAERFEDGTISFLDVIALKHGFDLRLTLTG--GMEINQKHTTLLAQTYTALSSLOYPNGAFVVRISYDSEFFSSPEVO--GPIINFNVLDDKGNIGYQVDKMASLYNIHRTGCFNPGACAKYLEL

Cattle (258) RKT-YFGGTAASAYLAGEDFYIPQ--SVAERFEDGTISFLDVIALKHGFDLRLTLTG--GMEINQKHTTLLAQTYTALSSLOYPNGAFVVRISYDSEFFSSPEVO--GPVISFNVLDDHGNVVGYSQVDKMASLNIHVRITGCFNPGACAKYLEL

Canton-S (264) QKRFFFGGTTINAYPHAMDYQRLR--TFHORYEDGLPLFLSVGLLEGFRTLERLVRPTDEFSTMERISRHVFLGAKYLEDQRLHHPNGEPLVKLYNKVGYQDKSRQ--GGIVAFNVRTESGSFVGFGIEACVAALHGLLRTGCFNIGACQYYLGL

mal-f1 (264) QKRFFFGGTTINAYPHAMDYQRLR--TFHORYEDGLPLFLSVGLLEGFRTLERLVRPTDEFSTMERISRHVFLGAKYLEDQRLHHPNGEPLVKLYNKVGYQDKSRQ--GGIVAFNVRTESGSFVGFGIEACVAALHGLLRTGCFNIGACQYYLGL

mal-1 (264) QKRFFFGGTTINAYPHAMDYQRLR--TFHORYEDGLPLFLSVGLLEGFRTLERLVRPTDEFSTMERISRHVFLGAKYLEDQRLHHPNGEPLVKLYNKVGYQDKSRQ--GGIVAFNVRTESGSFVGFGIEACVAALHGLLRTGCFNIGACQYYLGL

A.nidulans (260) KHKRFFFGGTTINAYPHAMDYQRLR--TFHORYEDGLPLFLSVGLLEGFRTLERLVRPTDEFSTMERISRHVFLGAKYLEDQRLHHPNGEPLVKLYNKVGYQDKSRQ--GGIVAFNVRTESGSFVGFGIEACVAALHGLLRTGCFNIGACQYYLGL

Ar. (390) SHSELRSNVE-AGHICWDDNDVINGKPTGAVRVSGYMSFEDAKKFDIFFISSFASPPK-----KTNGTVVSGRFPQLFSEDLSEKESFPS-----HYLKSITVYPIK

Tomato (339) SHLDLNSNIE-AGHVCWDDRDILHGKPTGAVRVSGYMSFEDAMKFNVESENFVIS-----SFNRCALQPRISLPIEGTA--EAAA--HFLTSTVYPIK

Human (388) SNEMVRKHQ-AGHVCDDNDLIDGPTGSVRISFGYMSLDDVQAFRLFIIDTRHSSGDWVFPQAHADTGETGAPSADSOAD-VIPAVMGRDRLSPQEDALTGSRVWNIS-STVNAVFPVAVPVDVARTQPTPEKAAGVLEGALGPHVVTNLYPIK

Cattle (356) SDEMVRKHQ-AGHVCDDNDLIDGPTGSVRISFGYMSLDDVQAFRLFIIDTRHSSGDWVFPQAHADTGETGAPSADSOAD-VIPAVMGRDRLSPQEDALTGSRVWNIS-STVNAVFPVAVPVDVARTQPTPEKAAGVLEGALGPHVVTNLYPIK

Canton-S (375) DEDALDAIYKRAGRICDYFDLIDGPTGAVRVSGYMTTIQDVLQQLMLRSSYLATKE--LQRIQFIEEQAEQLPPLLKER--VQLLREKLLQMA--IYVVK

mal-f1 (375) DEDALDAIYKRAGRICDYFDLIDGPTGAVRVSGYMTTIQDVLQQLMLRSSYLATKE--LQRIQFIEEQAEQLPPLLKER--VQLLREKLLQMA--IYVVK

mal-1 (377) DEDALDAIYKRAGRICDYFDLIDGPTGAVRVSGYMTTIQDVLQQLMLRSSYLATKE--LQRIQFIEEQAEQLPPLLKER--VQLLREKLLQMA--IYVVK

A.nidulans (360) TGADMLRHFS-AGMRCDDHDIMDERPTGILRISLGAMSSLTVDVTFIAFLFEFYVDKPF-----EGLFVPLTGNVSLHQPSFYVES-----LSVYPIK

Ar. (547) SCAGFSVIR--WPLCRTGLLHDEWVQGL-TGEILTQKKVPFMSLITFTDLEGLLSVESRCDKLRHKSDS-----YNPRNDEFDSHANILNRNEETRINRHTFNAIGRQCKLLYSSTSKDCLN

Tomato (507) SCAGFSVDQ--WPLSTGLLHDEWILKST-TGEILTQKKVPFMYSTITIDENLGLKLFVESPRCKEKLQIEKSSS-----LVTERDEMIDQNHRYEYTSYNNVDWFRRAIDRPTLLNNSDSQSHSCIN

Human (565) SCAAFEVTR--WPGVNGQLLYDRSMVNVH-NGVCLSKQOEPRCLIQEFTDLQRQIMVIKA-KGMEPIEVPLEENS-----ERQIQIRQSVCAVRVSTYDCGEKISNLSLTFFGFRPCNLIKQSSNSDRNAK

Cattle (503) SCAAFEVIR--WPLGSQGLLYDRSMVNVH-NGICLSKQOEPRCLIQEFTDLQRQIMVIKA-QGMEPIEVPLEENS-----EQVOICQSKVCADRVTYDCGEKISNLSLKFGRFVHLIKQSSDFORNAK

Canton-S (515) SCAAFKIELPGSWPLTDQGLKYDREWMIVDM-NGMALTQKRCFELCLIRPVLKVDQLELOFGE-NSTISVPLSLDDQA--ADTAKCVSKVCRQFVEGLDCGRVAQWLSENGMEGLRLLRQSG--QRNSS

mal-f1 (428) SCAAFKIELPGSWPLTDQGLKYDREWMITDQGLKYDREWMIDPETLHGVPVNP*-----ADTAKCVSKVCRQFVEGLDCGRVAQWLSENGMEGLRLLRQSG--QRNSS

mal-1 (517) SCAAFKIELPGSWPLTDQGLKYDREWMIVDM-NGMALTQKRCFELCLIRPVLKVDQLELOFGE-NSTISVPLSLDDQA--ADTAKCVSKVCRQFVEGLDCGRVAQWLSENGMEGLRLLRQSG--QRNSS

A.nidulans (544) SCAGFIRPDQGRWEVREGLAWDREWCILVHQTGITLNQKRYRPMALIRFTLDERCLLRITCGEANSRDGKTLEISLNIRIGTNSLTSLQCNASKPSTVCGDKVVLQAYTSPAVSRFFTFDLGVFECTLARFPQSSSTRFHSRATAANRDNQNSQKQSPS

Ar. (651) ---RMKSPGLCRDLESNINFANEAQFLLISEESVADLNRLEAKDEDYKR--AHEKLNPHFRPNLVISG--GEPYGEDKWKTKVIG--DNHFTSLGGCNRQCMINISNEAGLVKKSNEPLTLTASYRRVKGILFGTLRYEIDEKQ--C

Tomato (594) ---KNGSPGMRDVGARLNFVNEAQFLLISEESIKDLNRLKNSGRRNRNG--QAVQVGVMFRPNLVASS--GEPYADGDWSNNIG--GYFMSLGGCNRQCMINISNEAGVQRFTEPLATLAGYRAKGIKMGITLLRYENNTKTESDT

Human (604) ---KKGHDQLPGTMTATSLVNEAQYLLINTSSILELHQRLNTSDENGKEEL--FSLKDLSLFRANITING--KRAFEEEKWDEISIG--SLRFQVLGFCCHRCQMICIDQQTGOHNO--HVFOKLSERR--ETKVNFGMYLMHASLDSLSSPCF

Cattle (662) ---KKGHDQSAHTTATSLVNEAQYLLINRSSILELOQLSTSCENGKEEL--FPMNNLISFRANITING--TRAFFEEKWDEISIG--SLRFQVLGFCCHRCQMICIDQQTGOHNO--DVFOKLSERR--ERKVKFGVYLMHTSLDLSPPCY

Canton-S (671) ---KDQQA--SLVNAQAFLLINKSSVRSLOFEE--PLDETVDREFRANITIDT--GSAFEELTYKALSIG--GIQFQVEGFCQCRDMICINORTGERSP--ETLTTISRLQ--KGRMRFGIYITRIPQDT

mal-f1 (0) ---KDQQA--SLVNAQAFLLINKSSVRSLOFEE--PLDETVDREFRANITIDT--GSAFEELTYKALSIG--GIQFQVEGFCQCRDMICINORTGERSP--ETLTTISRLQ--KGRMRFGIYITRIPQDT

mal-1 (673) ---KDQQA--SLVNAQAFLLINKSSVRSLOFEE--PLDETVDREFRANITIDT--GSAFEELTYKALSIG--GIQFQVEGFCQCRDMICINORTGERSP--ETLTTISRLQ--KGRMRFGIYITRIPQDT

A.nidulans (662) MPGSFFQAPSSPDYPPTPILSNESPLLISRSSVNRNLNESIKSASQPCSNPGSAASKKAAVADVFRANVVAENISTAEPRYIEDTASLISGSGPEQLFDFLGSERCERQMVCDVDTGOAGD--EPYATLAKTRKIDRKILFGRIHSPVGRPKDAENG

Ar. (781) WIGVGEENVNIE-----WIRVGEETIENGDRH-----

Tomato (774) LSVGSQVLPVLKENVEGHLPASEKHQDVTS-----

Human (853) LSVGSQVLPVLKENVEGHLPASEKHQDVTS-----

Cattle (821) LSVGSQVLPVLKENVEGHLPASEKHQDVTS-----

Canton-S (782) ---KLEFKEQHMTCCGVVLVE-----

mal-f1 (0) ---KLEFKEQHMTCCGVVLVE-----

mal-1 (784) ---KLEFKEQHMTCCGVVLVE-----

A.nidulans (816) CLGTIMVGDAVTPSYDNES-----

4.6. Conclusions.

Prior to this study despite AO and XOR being classed as detoxification enzymes there is no published unequivocal proof of this.

In this study two mutant strains of *Drosophila melanogaster* were used as animal models to investigate this. Rosy (*ry*) strains have a deficiency in XOR, whilst maroon-like (*ma-l*) have a lack of functional AO/ XOR due to a MCS deficiency. Biochemical evaluation of these two strains by sensitive HPLC assays both *in vitro* and *in vivo* confirmed that the *ry* and *ma-l* strains were animal models for human hereditary xanthinuria type I and II respectively.

PCR cloning and DNA sequencing of the MCS gene in the maroon-like strains revealed that the *mal-1* and *mal-fl* strains had a 6 bp insertion and a 23 bp deletion in exon 4 respectively that are predicted to lead to changes of the deduced MCS protein sequence when compared to wild type sequence, thus explaining the deficiency in MFEs in these strains. Once again this reflects the situation found in humans where the dual deficiency is caused by mutations in the MCS gene.

Initial studies using spectrophotometric and HPLC assays indicated that MFEs in *Drosophila* were able to catalyse the biotransformation of numerous substrates of the well-characterised mammalian AO. These included the xenobiotics (such as dimethylcinnamaldehyde, phenanthridine and vanillin) and drugs (such as allopurinol, cinchonine and pyrazinamide). Endogenous vitamin substrates included retinal, pyridoxal and N¹-methylnicotinamide were also biotransformed by *Drosophila* AO.

Investigation of the MFEs in *ry*⁵⁰⁶ strain revealed a deficiency of enzyme activity to XOR substrates such as xanthine and the caffeine metabolite 1-methylxanthine. *Mal-1* and *mal-fl* strains were found to be unable to biotransform all AO substrates tested

table 37. Investigation of the enzyme activity in *ry* strain revealed a compromised ability to biotransform several XOR substrates that reflected the situation in human hereditary xanthinuria type I with allopurinol, N¹-methylnicotinamide, pyrazinamide and xanthine. These results confirmed that *Drosophila* mutants to be good experimental models for testing the role of MFEs as detoxification enzymes.

In order to test the role of the enzymes in chemoprotection MFE substrates were administered in *Drosophila* media and survivorship monitored. This demonstrated that several methylated xanthines including caffeine, theophylline and theobromine were toxic to XOR-deficient strains. In addition a range of AO substrates including N-heterocycles and aldehydes were toxic to *ma-l* AO-null strains (Table 37). This study therefore provides unequivocal proof that both AO and XOR are involved in detoxification.

Investigations to determine the effect of MFE deficiencies on lifespan revealed that *ry* and *ma-l* strains had significantly reduced lifespans when compared with the wild type strain, with the mean lifespan being reduced approximately 60% and 30% in the *ry* and *ma-l* strains respectively. The effect of MFE deficiencies on environmental heat and cold stress were also determined. This indicated that *ry* and *ma-l* strains had significantly reduced ability to survive at -3, 0, 37 and 39°C with the AO-null *ma-l* strain being most compromised in these conditions. The effect of the toxic chemicals, lifespan and temperature stress were rescued in *ry*⁵⁰⁶ animals that had the normal *ry* (XOR) gene reinserted by transgenesis.

4.7. Recommendations for future work.

Although it was proven that a deficiency of AO in maroon-like *Drosophila* strains resulted in the absence of cinchonine, pyrazinamide and N¹-methylnicotinamide biotransformation, the products of the reaction in AO-active strains were not definitively identified due to a lack of commercial availability of the metabolites. Had time permitted, experiments could have been optimised in order to unequivocally prove the products of these AO-mediated reactions were the same as those produced by their mammalian counterparts using LC-MS.

This study could be expanded to analyse a wider range of xenobiotics including drugs that are MFE substrates (Table 1 in introduction). Other chemicals that warrant attention in *Drosophila* as an experimental model are substrates that are biotransformed by the MFEs acting as reductases such as nitrated polycyclic hydrocarbons, zonisamide and doxorubicin (see tables 2 and 3 in the introduction for a list of other compounds). It would also be of interest to evaluate a range of other physical stresses for example ionising radiations such as gamma rays.

Although the reason for the reduced lifespan in *ry* flies is probably due to the lack of the antioxidant uric acid in this strain, the additional reduced lifespan in maroon-like strains is more difficult to comprehend. In addition, although it was conclusively proven that methylated xanthines such as caffeine and theophylline were significantly more toxic to MFE-deficient strains, the expected finding of an accumulation of toxic products was not apparent using HPLC.

There are several sophisticated advanced techniques that might be used to determine why the lack of AO in *Drosophila* leads to a decreased lifespan and low survivability at temperature extremes. These advanced techniques include metabolomics,

transcriptomics, proteomics that might reveal differences in metabolites, gene expression or protein expression between *ry* and *mal* strains and thus the molecular basis for this surprising observation. These techniques could also be utilised to determine the unresolved molecular basis of methyl xanthine toxicity in the mutant strains.

5. References

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6.0. Appendices

6.1. Appendix 1

Table showing the genetic code.

First base									
Second base	T			C			A		
T	TTT	Phe		CTT	Leu		ATT	Ile	
	TTC			CTC			ATC		
	TTA	Leu		CTA			ATA		
	TTG			CTG			ATG	Met	
C	TCT	Ser		CCT	Pro		ACT	Thr	
	TCC			CCC			ACC		
	TCA			CCA			ACA		
	TCG			CCG			ACG		
A	TAT	Tyr		CAT	His		AAT	Asn	
	TAC			CAC			AAC		
	TAA	STOP		CAA	Gln		AAA	Lys	
	TAG			CAG			AAG		
G	TGT	Cys		CGT	Arg		AGT	Ser	
	TGC			CGC			AGC		
	TGA	STOP		CGA			AGA	Arg	
	TGG	Trp		CGG			AGG		
	GTT	Val		GCT	Ala		GCT	Ala	
	GTC			GCC			GCC		
	GTA			GCA			GCA		
	GTG			GCG			GCG		
	GAT	Asp		GAT	Glu		GAT	Asp	
	GAC			GAC			GAC		
	GAA	Glu		GAA			GAA	Glu	
	GAG			GAG			GAG		
	GGT	Gly		GGT			GGT	Gly	
	GGC			GGC			GGC		
	GGA			GGA			GGA		
	GGG			GGG			GGG		

6.2. Appendix 2.

Single letter and triple letter codes for the amino acids.

Amino acid	Single letter code	Triple letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

6.3. Appendix 3.

%RSD for retention time (n=3)					
Analyte	0.01mM	0.02mM	0.03mM	0.05mM	0.1mM
1, 3-dimethylxanthine	1.87	2.31	2.23	2.14	1.79
1, 7-dimethylxanthine	1.19	1.49	2.58	2.11	1.61
1,3,7-trimethylxanthine	0.57	0.09	0.14	0.19	0.16
1,7-dimethyluric acid	1.61	2.08	1.57	2.01	1.66
1-methyluric acid	0.72	0.98	1.02	1.23	0.88
1-methylxanthine	1.61	1.25	1.86	1.02	0.61
3- methylxanthine	2.76	1.68	1.15	0.71	0.54
3,7- methylxanthine	1.87	3.05	1.63	1.19	2.08
6-mercaptopurine	2.51	0.68	0.39	1.11	0.17
6-thioxanthine	1.51	2.22	0.35	0.16	2.32
7-methylxanthine	3.31	1.32	0.55	1.16	0.89
Allopurinol	0.61	1.23	2.26	0.22	1.28
Cinchonine	1.66	1.68	0.15	2.11	0.74
Methotrexate	0.97	1.01	1.12	1.19	0.27
N ¹ -methylnicotinamide	1.12	2.13	0.52	0.27	1.66
Oxipurinol	0.87	2.05	1.83	0.59	1.18
Phenanthridine	2.25	3.17	1.13	0.22	0.78
6(5H)-phenanthridone	2.41	2.14	2.17	1.21	0.26
Pyrazinamide	1.12	1.51	0.57	1.42	1.29
Pyridoxal	1.41	0.18	0.24	0.89	1.86
Pyridoxic acid	2.14	0.77	0.26	1.13	0.66
Uric acid	1.22	1.53	0.52	1.37	2.26
Vanillic acid	0.77	1.61	0.89	0.35	0.59
Vanillin	1.02	2.23	0.42	0.25	1.16
Xanthine	1.21	0.98	0.19	0.11	1.79

Table 2: % RSD of retention time of three replicate runs of analytes used in this study in the same day of calibration standard for intra- day precision. The % RSD for retention time between injections is in the range of 0.09-3.31.

Mean \pm SD of accuracy (%) (n=3)					
	Nominal concentration				
Analyte	0.01 mM	0.02 mM	0.03 mM	0.05 mM	0.1 mM
1-methylxanthine	98.5 \pm 2.5	100.3 \pm 0.9	99.8 \pm 2.3	98.1 \pm 0.7	99.9 \pm 0.2
1,3,7-trimethylxanthine	98.2 \pm 2.2	99.8 \pm 1.7	100.4 \pm 0.2	99.9 \pm 0.2	98.8 \pm 1.2
1, 3-dimethylxanthine	100.5 \pm 0.3	98.7 \pm 0.5	98.1 \pm 1.2	99.9 \pm 0.4	99.8 \pm 0.7
1, 7-dimethylxanthine	99.7 \pm 1.1	95.2 \pm 2.2	99.8 \pm 0.7	97.7 \pm 1.6	99.9 \pm 0.5
1-methyluric acid	95.1 \pm 1.1	97.3 \pm 0.4	96.2 \pm 0.9	99.9 \pm 0.2	96.7 \pm 0.7
3- methylxanthine	99.9 \pm 1.3	99.7 \pm 0.7	99.8 \pm 1.8	98.6 \pm 1.5	97.9 \pm 1.1
1,7-dimethyluric acid	95.5 \pm 1.4	97.7 \pm 1.3	99.8 \pm 1.2	99.9 \pm 0.1	99.5 \pm 1.4
3,7-dimethylxanthine	98.7 \pm 2.1	98.9 \pm 1.1	99.5 \pm 1.5	99.6 \pm 0.3	99.8 \pm 1.1
6-mercaptopurine	98.2 \pm 0.3	97.2 \pm 1.6	95.5 \pm 1.7	98.8 \pm 1.1	99.2 \pm 1.7
6-thioxanthine	100.2 \pm 0.2	98.4 \pm 2.3	98.5 \pm 1.1	99.8 \pm 1.9	98.2 \pm 1.3
7-methylxanthine	98.3 \pm 0.9	99.8 \pm 2.1	99.7 \pm 1.2	99.6 \pm 1.3	98.1 \pm 1.3
Allopurinol	98.2 \pm 1.2	99.2 \pm 1.1	97.1 \pm 0.7	98.5 \pm 1.3	98.5 \pm 1.7
Cinchonine	97.5 \pm 1.5	98.3 \pm 0.8	97.8 \pm 1.4	105.6 \pm 0.2	96.9 \pm 1.2
Methotrexate	99.3 \pm 1.3	97.1 \pm 1.7	98.9 \pm 0.5	96.1 \pm 2.4	98.9 \pm 1.8
N ¹ -methylnicotinamide	95.1 \pm 0.7	98.5 \pm 0.4	99.9 \pm 0.4	100.3 \pm 0.7	96.8 \pm 1.4
Oxipurinol	99.2 \pm 2.1	99.1 \pm 1.1	100.4 \pm 2.2	96.6 \pm 1.9	98.8 \pm 2.1
Phenanthridine	100.6 \pm 1.9	99.8 \pm 1.4	99.2 \pm 0.3	99.1 \pm 1.3	100.8 \pm 1.1
6(5H)-phenanthridone	99.5 \pm 0.7	97.5 \pm 0.2	99.5 \pm 1.7	99.5 \pm 1.1	99.5 \pm 0.8
Pyrazinamide	100.5 \pm 0.4	97.3 \pm 1.3	99.9 \pm 1.1	99.3 \pm 1.2	98.3 \pm 2.2
Pyridoxal	98.2 \pm 0.7	97.9 \pm 1.6	95.9 \pm 2.3	99.9 \pm 0.6	100.2 \pm 1.7
Pyridoxic acid	98.4 \pm 1.5	95.4 \pm 0.7	99.1 \pm 2.4	98.2 \pm 1.8	99.2 \pm 1.3
Uric acid	95.6 \pm 1.7	99.7 \pm 1.1	99.7 \pm 1.2	98.3. \pm 1.9	97.2 \pm 0.7
Vanillic acid	97.1 \pm 1.7	98.5 \pm 1.8	100.5 \pm 0.4	97.5 \pm 1.3	99.1 \pm 0.9
Vanillin	97.1 \pm 1.2	99.1 \pm 1.1	98.8 \pm 1.1	95.2 \pm 1.7	98.5 \pm 0.7
Xanthine	97.4 \pm 1.2	99.6 \pm 1.3	100.2 \pm 0.1	99.8 \pm 0.7	99.6 \pm 2.1

Table 3: % Accuracy calculated for intra-day analysis of compounds used in HPLC analysis in this study.

Mean area of three injections in the same day of calibration standards (n=3)										
Analyte	0.01 (mM)	0.02 (mM)	0.03 (mM)	0.05 (mM)	0.1 (mM)	Slope	Intercept	RSQ	LOD (nmol)	LOQ (nmol)
1,3,7-trimethylxanthine	329073	588050	900438	1565542	3196561	16008	- 23976	0.994	0.004	0.023
1, 3-dimethylxanthine	296357	485573	803416	1386088	2584663	12984	17166	0.997	0.005	0.024
1,7-dimethyluric acid	350269	736064	1075433	1735800	3347587	16676	40207	0.992	0.002	0.014
1, 7-dimethylxanthine	200588	382509	578603	953698	1951522	9733	- 3526	0.998	0.0001	0.0005
1-methyluric acid	307169	539389	835522	1431411	2952992	14769	- 22718	0.998	0.001	0.005
1-methylxanthine	134653	265966	408825	648214	1250231	6228	15347	0.994	0.004	0.021
3,7-dimethylxanthine	326558	542411	881882	1519827	3156397	15821	- 36283	0.998	0.005	0.011
3- methylxanthine	220630	380331	617750	1048771	2157284	10813	- 19424	0.995	0.007	0.034
6-mercaptopurin	39438	79514	139407	288320	591803	6091107	-23441	0.994	0.004	0.02
6-thioxanthine	51175	92429	134115	259511	485648	4904335	-1172	0.997	0.002	0.01
7-methylxanthine	212000	343158	573379	994966	2037038	10230	- 22702	0.997	0.002	0.01
Allopurinol	14726	29588	49432	96833	188236	1926912	-4388	1.00	0.003	0.015
Cinchonine	99785	151658	197633	390061	889728	8843599	-21381	0.990	0.0002	0.001
N ¹ -methylnicotinamide	44454	91846	169578	308302	661872	6764775	-24091	0.996	0.001	0.005
Methotrexate	128570	171092	276739	493204	998593	9945386	-3388	0.997	0.0006	0.003
Oxipurinol	23394	47904	76786	129660	233588	2714	2357371	1.00	0.001	0.005
Phenanthridine	571679	992255	1180187	2130945	4222854	41430984	66235	0.997	0.002	0.009
6(5H)-phenanthridone	132015	260381	397202	689057	1322123	13294923	1474	0.999	0.002	0.01
Pyridoxic acid	31017	50616	87722	138318	230686	2304745	9060	0.987	0.002	0.01
Pyridoxal	28160	45701	77618	112825	202247	1944019	7967	0.992	0.002	0.095
Pyrazinamide	70181	148691	217140	331506	640131	6337483	12796	0.998	0.004	0.02
Uric acid	482658	1095004	1494725	2563976	4955041	24777	30879	0.999	0.003	0.015
Vanillic acid	38737	99515	181284	281098	553041	5617796	-4343	0.997	0.0001	0.0005
Vanillin	54118	146188	211879	378212	734053	7447551	-6589	0.999	0.002	0.01
Xanthine	123857	253547	406308	642818	1253249	6273	7542	0.995	0.004	0.02

Table 4: Regression calculation based on mean area of three replicates in the same day of MFE substrate and product calibration standards against the actual concentration.

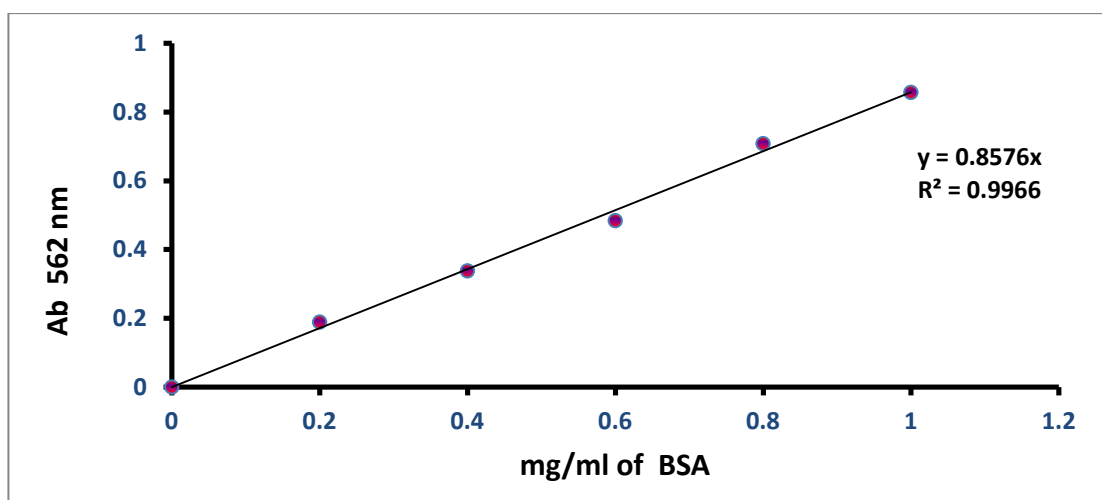


Figure 1: Standard curve for Bicinchoninic acid method obtained from known concentration of bovine serum albumin. The standard curve can then be used to calculate the protein concentrations of unknown sample.

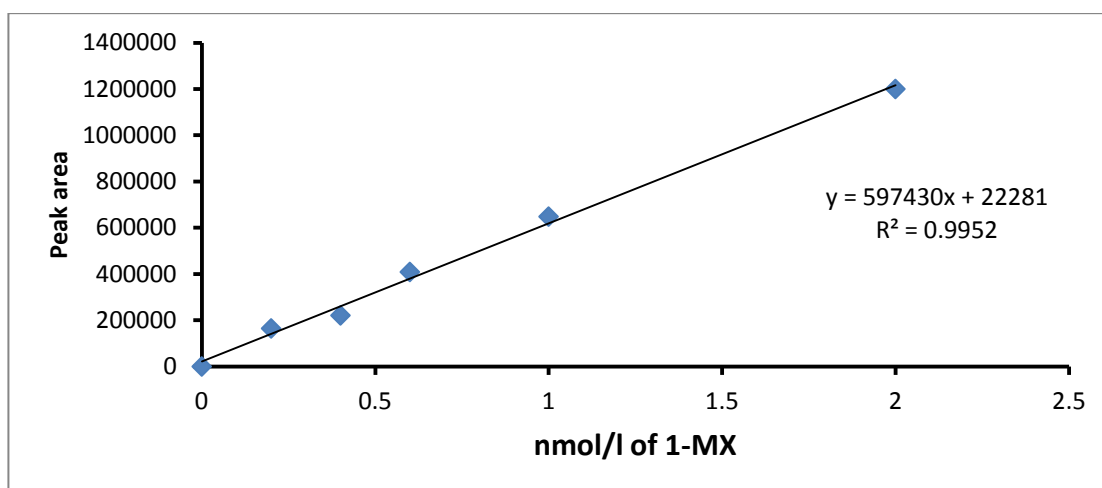


Figure 2: 1-methylxanthine standard curve.

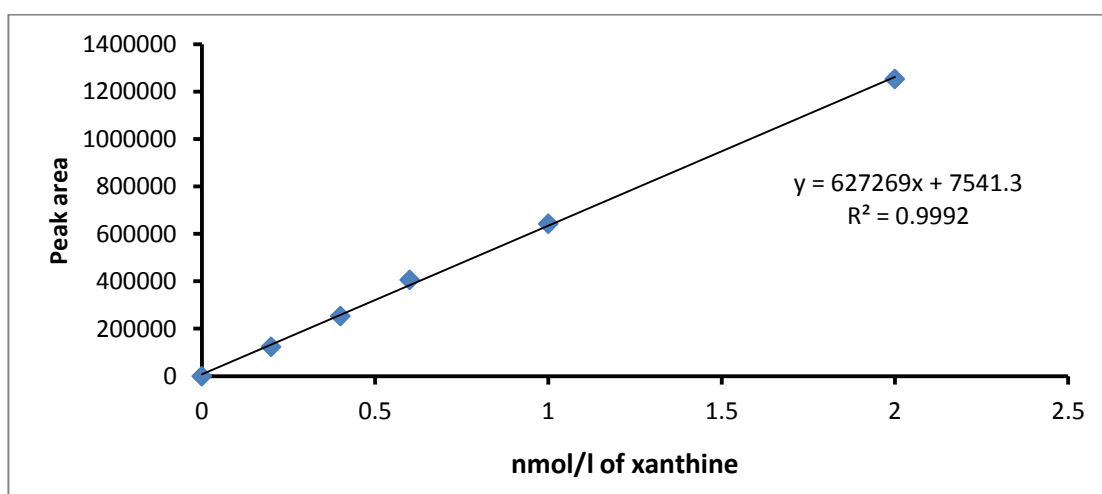


Figure 3: Xanthine (X) standard curve.

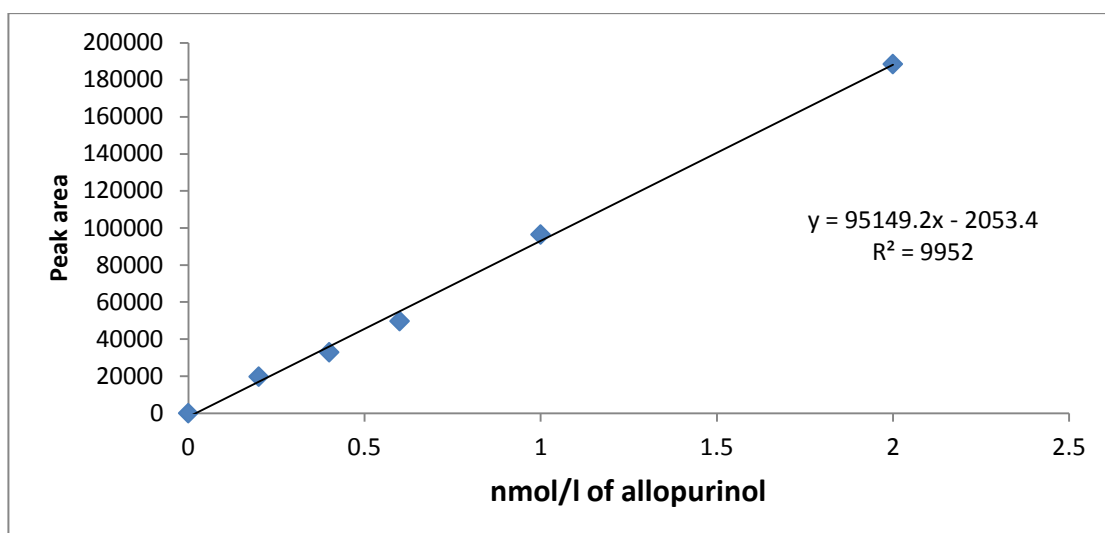


Figure 5: Allopurinol standard curve.

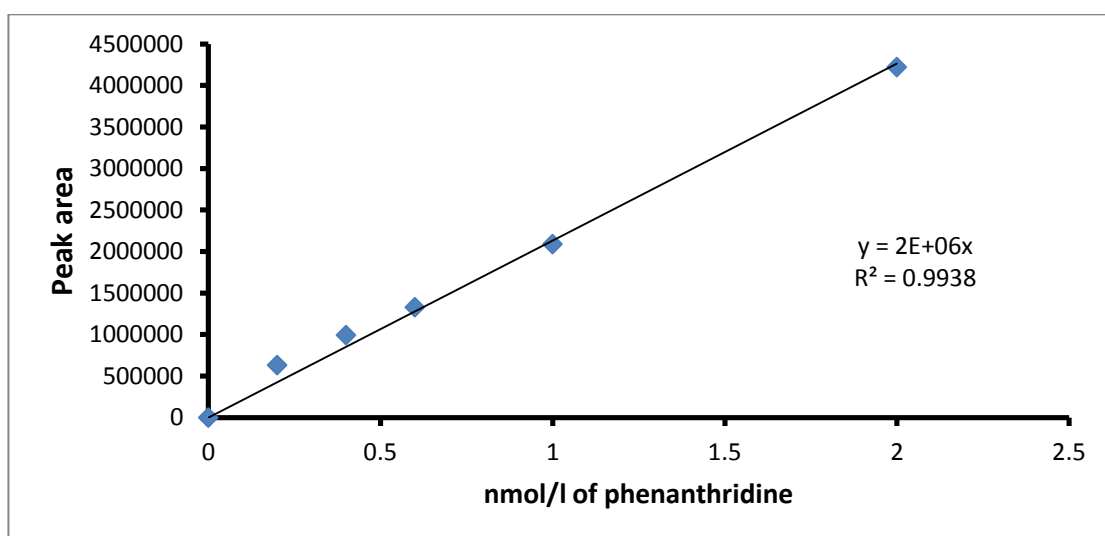


Figure 4: Phenanthridine standard curve.

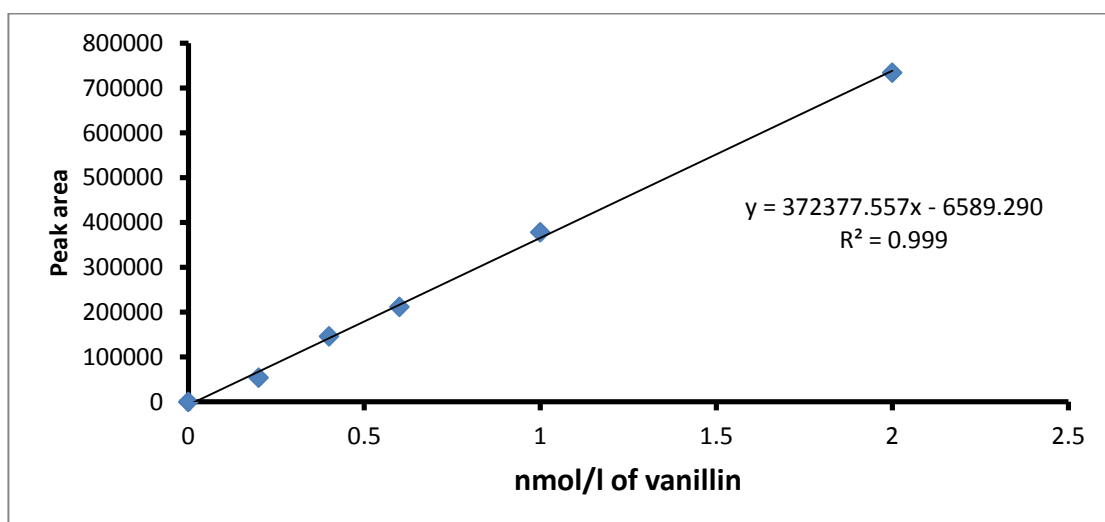


Figure 4: Vanillin standard curve.

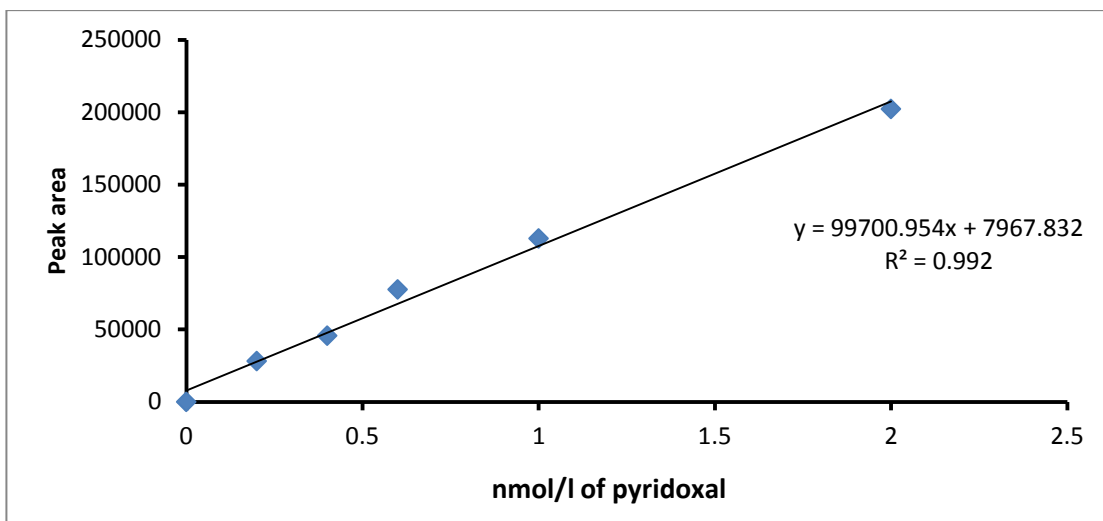


Figure 6: Pyridoxal standard curve.

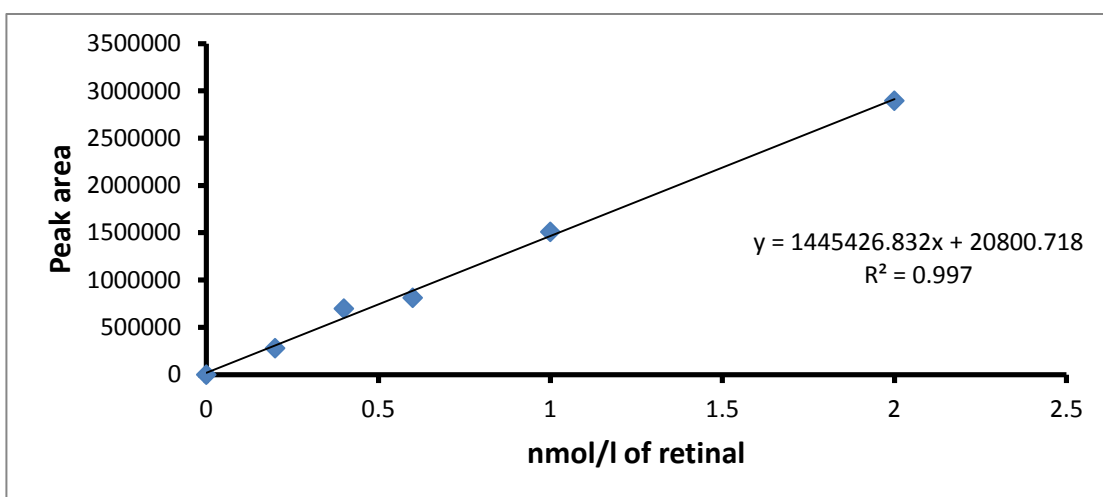


Figure 7: Retinal standard curve.

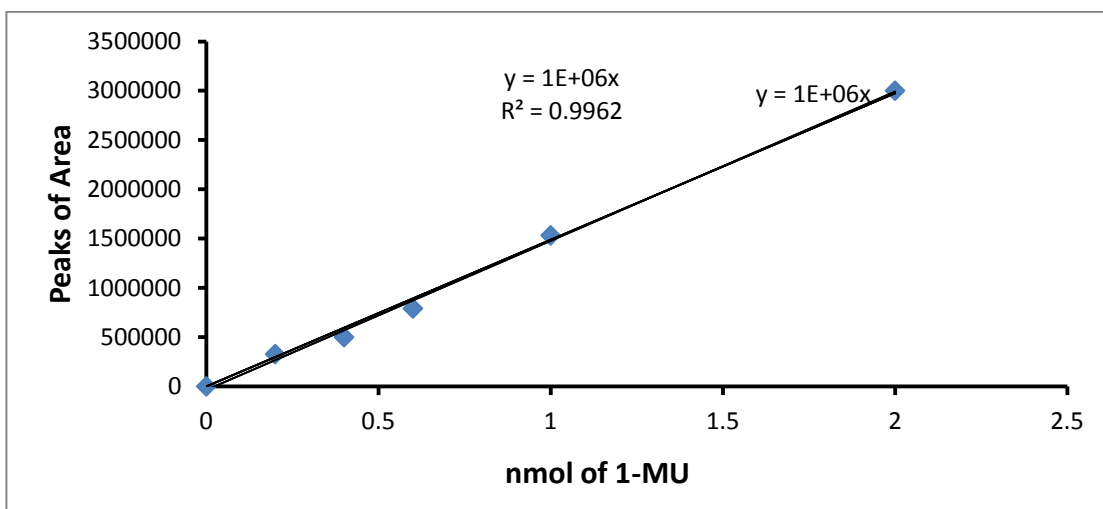


Figure 8: 1- methyluric acid standard curve.

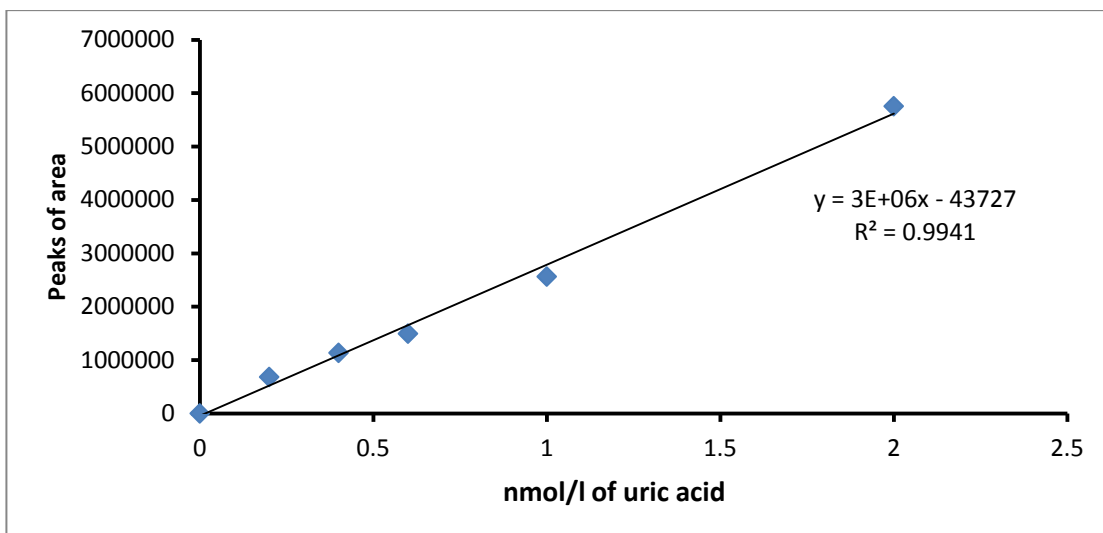


Figure 9: Uric acid (U) standard curve.

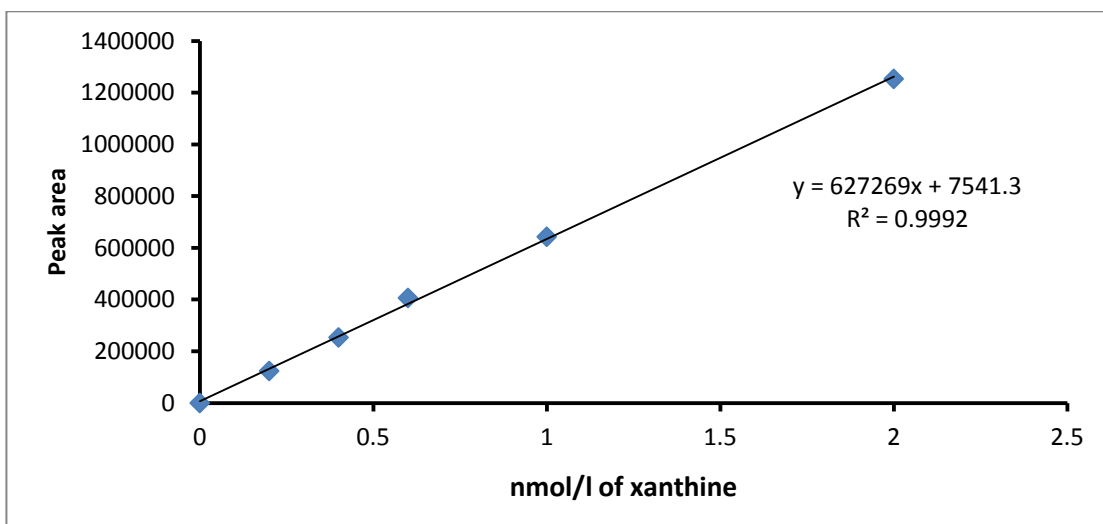


Figure 10: Xanthine (X) standard curve.

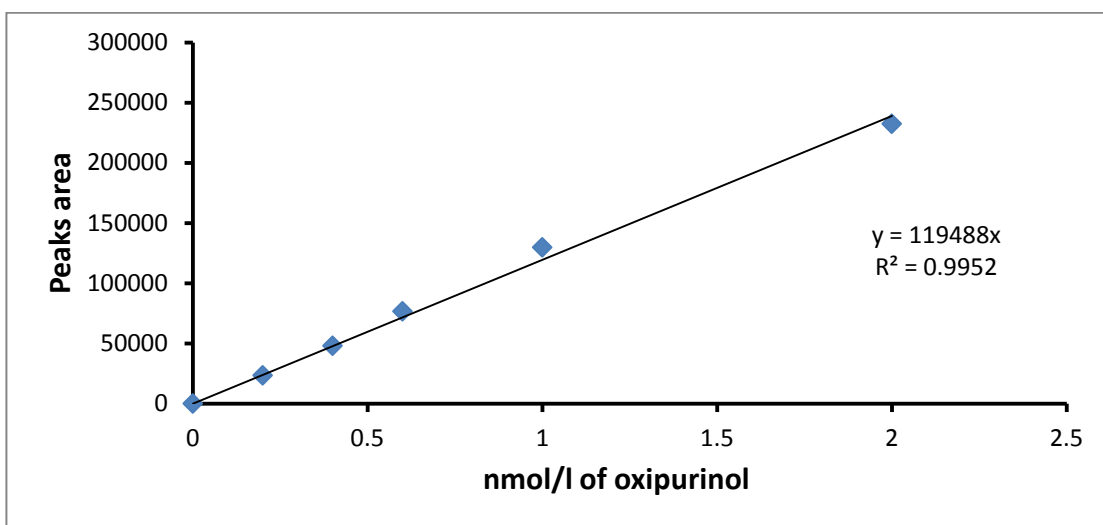


Figure 11: Oxipurinol standard curve.

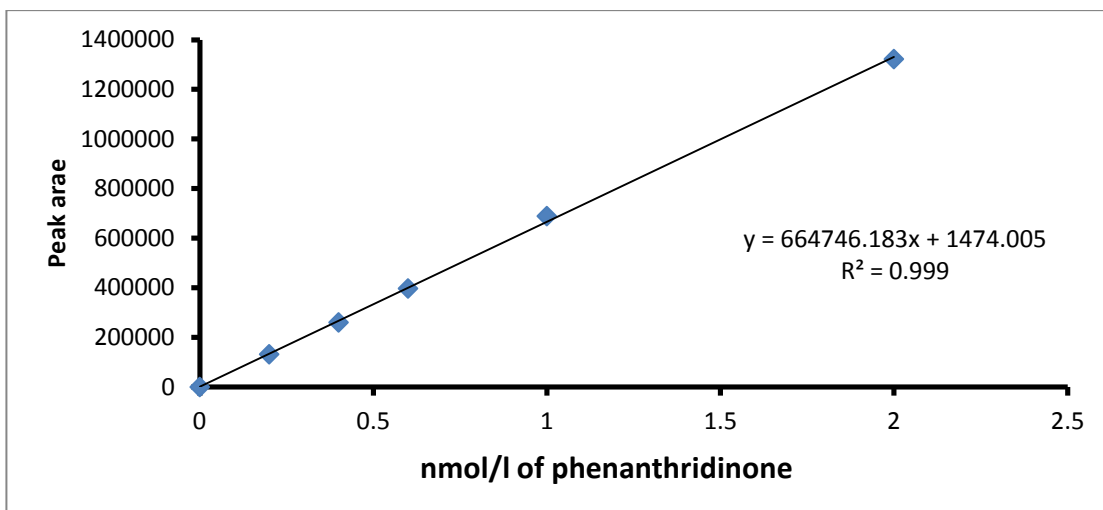


Figure 12: Phenanthridinone standard curve.

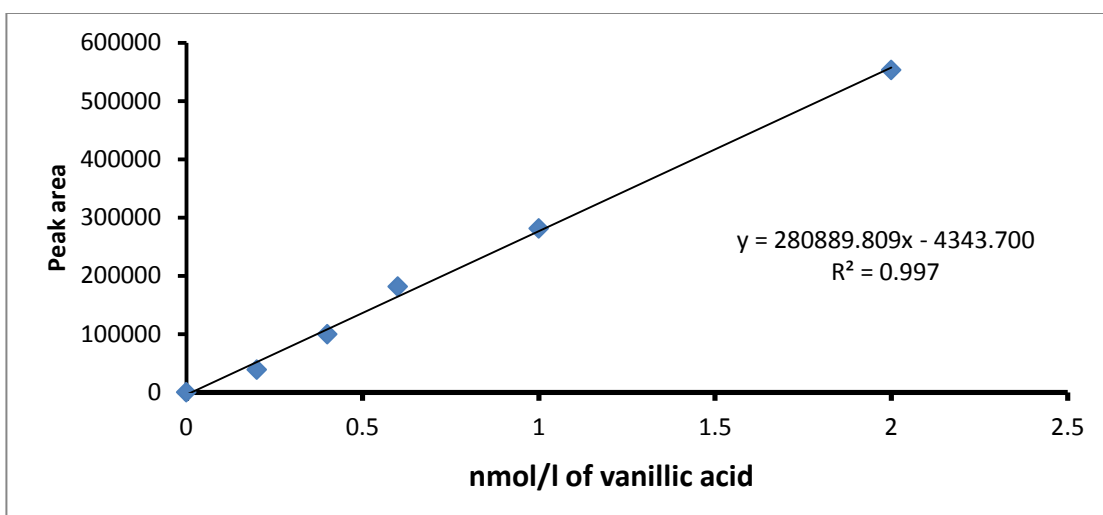


Figure 13: Vanillic acid standard curve.

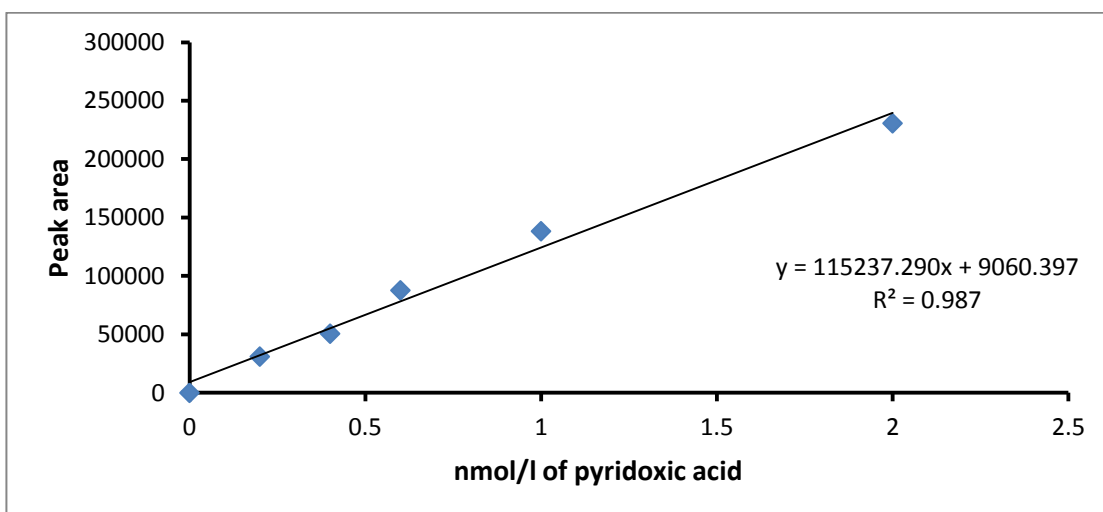


Figure 14: Pyridoxic acid standard curve.

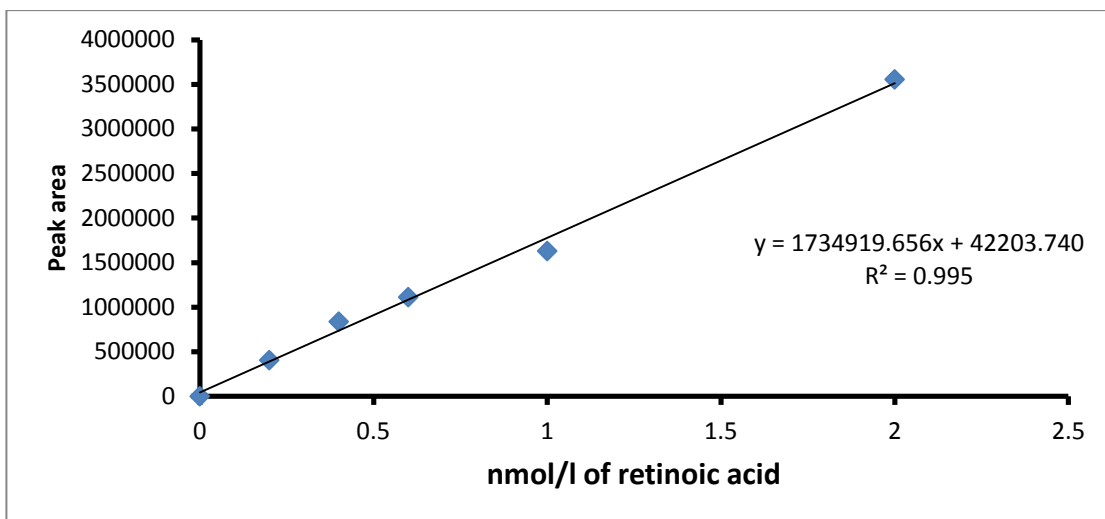


Figure 15: Retinoic acid standard curve.

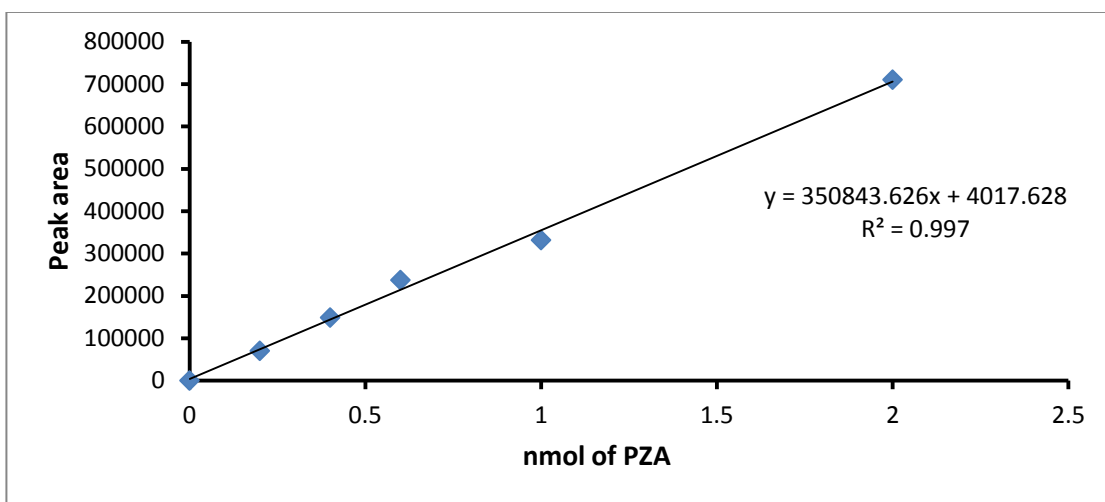


Figure 16: Pyrazinamide standard curve.

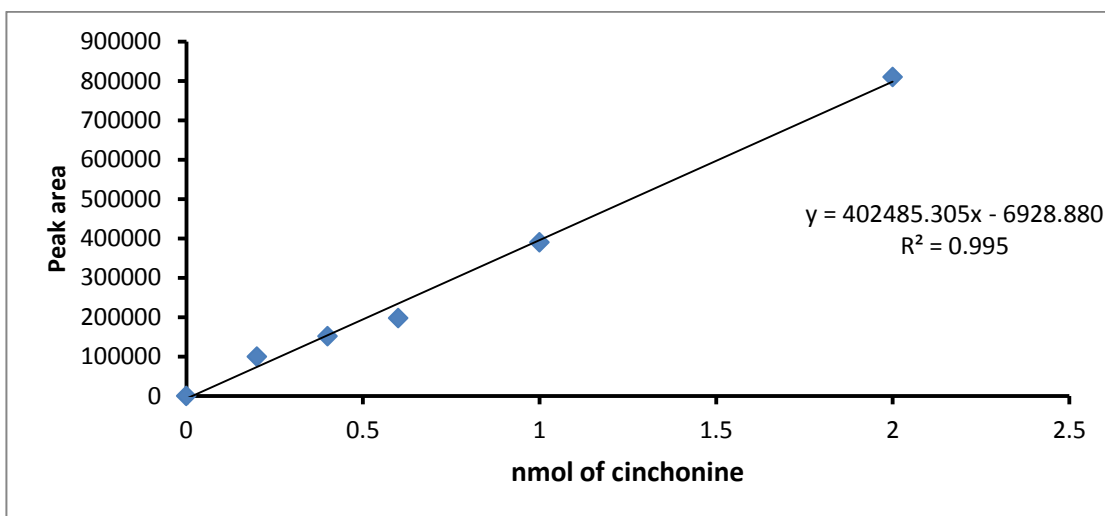


Figure 17: Cinchonine standard curve.

6.4. Appendix 4.

	Temperature(°C)			
	25	37	39	Total χ^2 value
Observed(O)	100	49.4	36.5	
Expected(E)	100	100	100	
O-E	0	-50.6	-63.5	
(O-E) ²	0	2560.3	4032	
(O-E) ² /E	0	25.60	40.32	65.92*

Table 1: Statistical difference in survival of wild type (Canton-S) strain after exposure to different heat shock temperatures at 6 hours. (* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

	Temperature (°C)			
	25	0	-3	Total χ^2 value
Observed(O)	100	58	13	
Expected(E)	100	79	55.5	
O-E	0	-21	-41.5	
(O-E) ²	0	441	1722.25	
(O-E) ² /E	0	4.41	12.22	21.63*

Table 2: Statistical difference in survival of wild type (Canton-S) strain after exposure to different cold shock temperatures at 4 hours. (* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value.

Xenobiotics and χ^2 value	Strain						
	Canton-S					<i>ry</i> ⁵⁰⁶	
	T1	T2	<i>ry</i> ⁵⁰⁶	<i>mal-1</i>	<i>mal-f1</i>	<i>mal-1</i>	<i>mal-f1</i>
6-Mercaptopurine (20 mM) χ^2 =	0.04	0.02	4.14*	4.94*	4.68*	4.94*	4.68*
Allopurinol (100 mM) χ^2 =	0.0001	0.0001	13.42*	-	-	-	-
Allopurinol (20 mM) χ^2 =	0.02	0.01	0.58	27.22*	27.17*	27.22*	27.17*
Caffeine (20 mM) χ^2 =	0.02	0.01	18.42*	26.66*	12.84*	26.66*	12.84*
Cinchonine χ^2 =	0.07	0.08	0.02	8.28*	9.85*	7.21*	8.01*
Oxipurinol (20 mM) χ^2 =	0.0001	0.0001	25.81*	37.35*	32.35*	37.35*	32.35*
Paraxanthine (100 mM) χ^2 =	0.004	0.02	18.65*	17.94*	20.97*	17.94*	20.97*
Phenanthridine χ^2 =	0.002	0.0002	0.001	49.29*	50.28*	48.72*	47.12*
Pyrazinamide χ^2 =	0.0001	0.0006	27.236*	36.83*	37.69*	5.83*	5.51*
Theobromine (20 mM) χ^2 =	0.001	0.002	24.24*	24.38*	24.55*	24.38*	24.55*
Theophylline (20 mM) χ^2 =	0.01	0.01	9.92*	13.69*	12.67*	13.69*	12.67*
N ¹ -Methylnicotinamide χ^2 =	0.003	0.004	0.006	45.75*	46.13*	45.17*	44.82*
Vanillin χ^2 =	0.00	0.00	0.00	57.02*	59.24*	59.75*	60.08*

Table 3: Statistical difference in survival between wild type, transgenic and mutant strains after exposure to xenobiotics. (* $P < 0.05$ by χ^2 test is 3.84) indicates when a value is significantly different from the control value. - : not tested